INHIBITORS OF BACTERIAL SPORE OUTGROWTH

BY

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THESIS PRESENTED FOR THE DEGREE DOCTOR OF PHILOSOPHY

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September 1981.

FOR CAROLYN

CONTENTS

Acknowledgements

I would like to thank the following people: Dr. Ian Dawes who by example and with kind consideration, encouragement and advice, both in and out of the laboratory, has made the tenure of my studentship a more complete education and a pleasure. Similarly I thank Professor Grahame Gould and Gerry Dring of Unilever Research for their time and interest. Dr. John Wright for his companionship, patience and hours of stimulating discussion over many aspects of my work. Moira, who typed this report. Professor J.F. Wilkinson for permission to work in his department. Many other people for varied assistance and advice, in particular Noni Ajam, Geoff Calvert, Gordon Cumming, Steve Emsley, Mrs. Lesley Hall, Dr. I.W. Sutherland and Dr. C. Whitfield. The hundreds of Salmo salar and Cyprinus carpio whose indifference to my lures and baits has enabled me to devote more thought to my work than I would have liked. Last but by no means least my wife, Carolyn, for her devotion, sacrifice and as a constant reminder that there is more to life than Bacillus subtilis spores. I would also like to acknowledge the financial support of a CASE studentship from the SERC and Unilever Research .

Declaration

I hereby declare that this thesis has been composed by myself and that the research presented, is, to the best of my knowledge, my own work. Due acknowledgement is made within the text for contributions from other sources.

K.T. Smith, September 1981

Abstract.

In order to understand controls which operate during outgrowth it is necessary to identify events which differ between outgrowing spores and vegetative cells. To this end the nature of the preferential inhibition of spore outgrowth by chloroquine has been investigated. The drug inhibited outgrowth of spores of several Bacillus spp. at lower concentrations than those preventing vegetative growth. Chloroquine intercalates with DNA and the combined action of chloroquine and, another intercalating molecule, ethidium bromide, on B. subtilis spore outgrowth was synergistic. However, the inhibition of outgrowth by chloroquine was independent of the initiation of DNA replication and of the synthesis of DNA, and therefore chloroquine was not acting on these processes per se. Analysis of macromolecular synthesis in outgrowing spores and in vegetative cells in the presence of chloroquine indicated that it acted preferentially on transcription. The basis of differential sensitivity of outgrowing spores may, therefore, have been a reflection of changes in RNA polymerase. However, RNA polymerase activity measured in permeabilized vegetative cells and outgrowing spores was inhibited to the same extent by chloroquine.

In the absence of any detectable changes related to chloroquine inhibition in the transcriptional machinery during outgrowth, factors affecting the uptake and accumulation of the drug were investigated. The preferential inhibition of spore outgrowth was not evident at pH8 at which chloroquine is to a greater extent in the monovalent, more lipophilic form. In the presence of inhibitors affecting energy metabolism vegetative cells were as sensitive to chloroquine as outgrowing spores. Measurement of the accumulation of [^{14}C] - chloroquine showed that early outgrowing spores accumulated twice as much drug as outgrowing spores that had escaped from inhibition by low levels of chloroquine and seven times more than vegetative

cells. Vegetative cells treated with metabolic inhibitors accumulated as much chloroquine as early outgrowing spores.

An hypothesis to explain the preferential inhibition of spore outgrowth by chloroquine is presented. In this model outgrowing spores accumulate more drug as result of the known low internal pH of spores. The lipophilic form of chloroquine enters the cytoplasm, ionizes to the less lipopilic form and is unable to leave the spore. Vegetative cells have an energy-dependent mechanism which effectively causes efflux of chloroquine by reducing the formation of the less lipophilic formof chloroquine. This mechanism is absent during early outgrowth. Furthermore, a mutant resistant to chloroquine during vegetative growth has been isolated and a membrane protein of molecular mass 260,000 has been shown to be derepressed in this mutant. The time at which outgrowing spores escape from inhibition by chloroquine has been correlated with that for the appearance of this high molecular mass protein during outgrowth. This protein may, therefore, be related to the development of an energized membrane during outgrowth.

Chapter 1

Introduction

I. Introduction

The mechanisms by which various cell processes are controlled are a fascinating but often elusive prey for biologists. In trying to understand the most complex forms of control, those acting during differentiation and morphogenesis, endospore forming bacteria of the genus Bacillus have been extensively studied. The synchronous nature of the processes of endospore formation and germination and spore outgrowth, make them ideal organisms for studies concerned with differentiation and its control. In the following discussion the biochemistry and genetics of spore outgrowth are considered in relation to the possible control mechanisms which may operate. The related processes of sporulation and germination are of considerable interest in themselves but for the most part are beyond the scope of this introduction, and very good recent reviews covering control, morphogenesis and genetics of sporulation (Dawes & Hansen, 1972; Piggot & Coote, 1976; Sonenshein & Campbell, 1978) and germination (Gould, 1969; Gould & Dring, 1972; Smith, Moir & Lafferty, 1976; Keynan, 1978) are available.

During the life-cycle of a typical Bacillus species (Figure 1), the vegetative cell, in response to particular starvation conditions, initiate a diverse but ordered sequence of biochemical and morphological events leading to the formation of a dormant, highly resistant endospore. On resuspending these dormant spores in a buffered solution containing one or more low molecular weight molecules a sequence of reactions is triggered and the spore germinates. Germination is essentially a degradative process involving hydration, excretion of components specific to spores e.g. dipicolinic acid (DPA) and (for B. subtilis) sulpholactic acid and various structures and enzymes are activated or unmasked. In general,

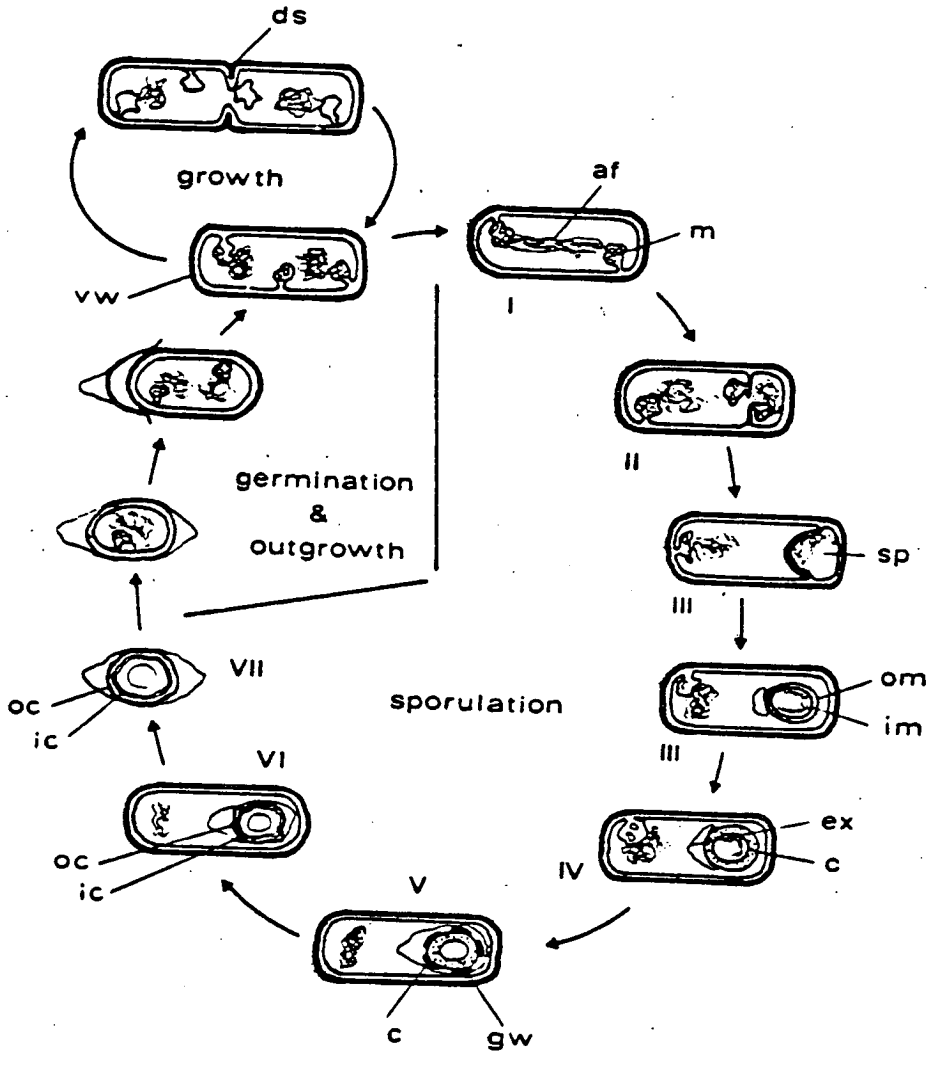


Figure 1. Main morphological changes occurring during morphogenesis in Bacillus species from Dawes & Hansen (1972). The following symbols have been used: vw, vegetative cell wall; ds, division septum; af, axial filament; om, outer forespore membrane; im, inner forespore membrane; m, mesosome; ex, exosporium; c, cortex; gw, germ cell wall; ic, inner spore coat; oc, outer spore coat; and sp, spore protoplast.

germination is completed within about 15 minutes for some strains e.g. B. cereus and 60 minutes for others e.g. B. subtilis. In response to suitable nutrients, the resulting metabolically active spores proceed through a biosynthetic stage of outgrowth to become vegetative cells.

Outgrowth is customarily defined as the stage after germination, beginning at post-germinative swelling and the onset of biosynthesis, up to the first cell division (Strange & Hunter, 1969). Although this is a convenient definition it is possible that the cell is not completely vegetative until several cell divisions have occurred (Goldman & Blumenthal, 1960).

2. Biochemistry of Outgrowth

Outgrowth is similar to sporulation in that there are several ordered morphological and biochemical stages, typical of a differentiating system, during which various parameters can be measured (Strange & Hunter, 1969; Dawes & Hansen, 1972; Keynan, 1973). Unlike sporulation with seven arbitrarily defined; but fairly distinct stages, outgrowth has only been divided into three less-well defined morphological stages, those of swelling, emergence from the spore coat and elongation (Figure 2). At present it is difficult to construct a time-scale for these outgrowth stages, unlike those for sporulation (Dawes et al., 1969) and events are usually related to the start of ribonucleic acid (RNA) and protein synthesis. Synchrony of outgrowth in a population of spores is never complete, Bacillus cereus T has a greater rate and therefore synchrony of germination than B. subtilis and is often the organism of choice in biochemical studies, although more extensive genetic techniques are available for the latter organisms. Ageing of the spore preparation, heat activation and pre-germination in a medium unable to support outgrowth may alleviate problems caused by asynchronous germination.

Spore outgrowth is one of the few examples in which a biosynthetically inactive structure becomes active. In order to understand the biochemical basis of this differentiation, it is necessary to identify and assess the relevance of the processes which occur. Figure 2 correlates the major biosynthetic events and the morphological changes of the outgrowing spore. Briefly, during swelling, water and nutrients are absorbed, RNA synthesis begins at a low rate, cell wall material is synthesized and protein synthesis is initiated. On emergence of the cell from the spore coat, the rates of RNA and protein synthesis increase rapidly

and net DNA synthesis begins, elongation is a continuation of high rates of macromolecular synthesis leading eventually to an actively dividing cell (Strange & Hunter, 1969; Keynan, 1973).

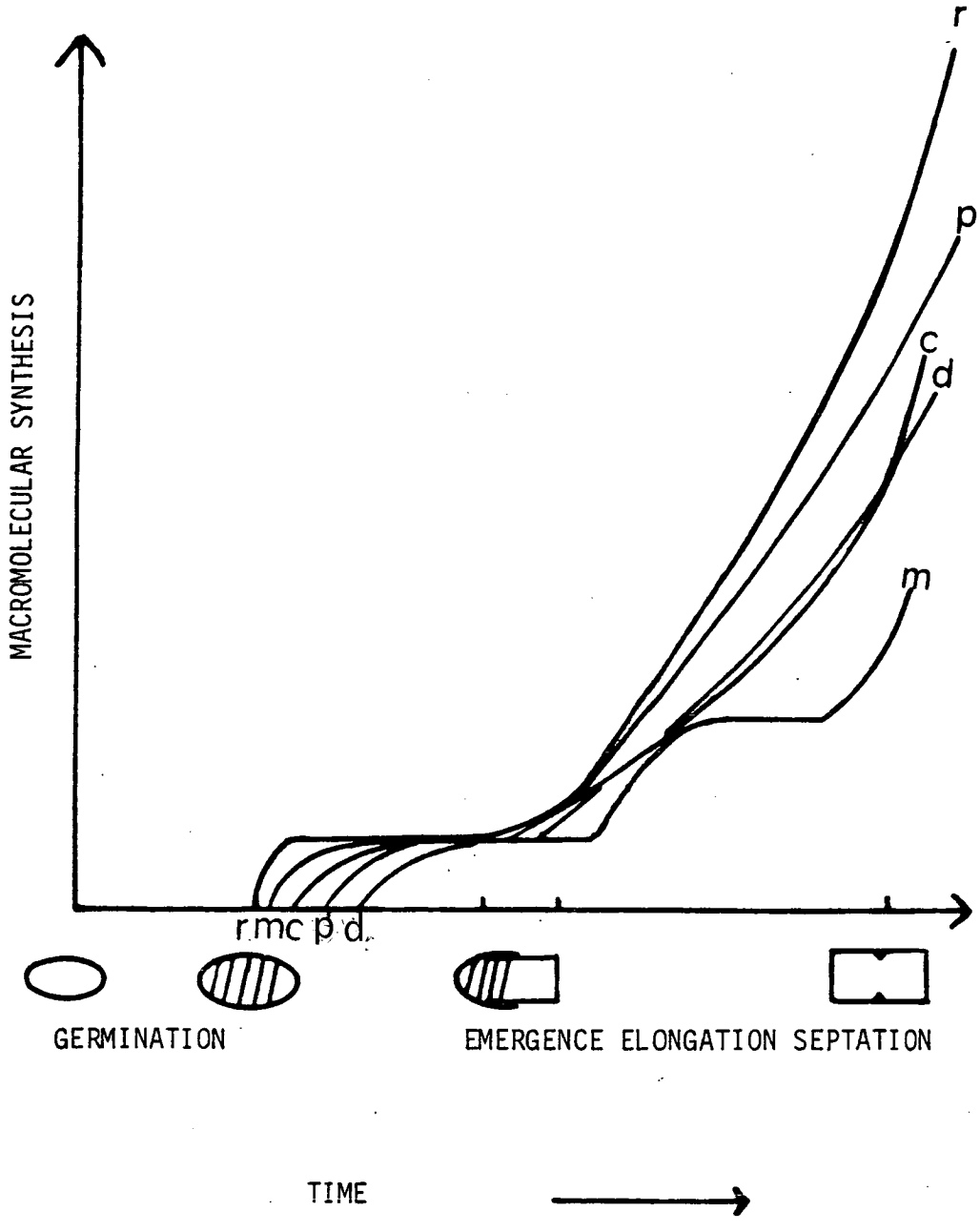


Figure 2. Relationship between the main macromolecular biosyntheses and morphological changes during outgrowth of Bacillus spores. The following symbols have been used: r, RNA; m, membrane lipids; c, cell wall; d, DNA; and p, protein.

2.1 Energy metabolism during outgrowth

The events shown in Figure 2 require metabolic energy but dormant spores only contain about 0.4% of the amount of adenosine-triphosphate (ATP) found in vegetative cells, similarly many other high energy compounds are present only in low amounts (Nelson & Kornberg 1970; Setlow & Kornberg, 1970a). Where does the spore obtain the energy required to initiate biosynthesis? Table 1 shows the levels of various nucleotides and metabolites associated with energy metabolism that are found in dormant spores and vegetative cells of Bacillus megaterium, the organism most widely used to study energy metabolism during germination and outgrowth. The levels of total adenine nucleotides, other ribonucleotides and pyridine nucleotides are very similar to those found in vegetative cells. The spore does contain high levels of 3-phosphoglyceric acid (PGA) sufficient to convert the nucleotides present to the triphosphate forms several times over (Setlow & Kornberg, 1970a). During germination ATP levels increase by 150 fold and are maintained at this level for about 15 min. All of this increase in the amount of ATP is supported from endogenous reserves and can occur in the absence of nutrients, these reserves seem likely to include PGA, since its concentration decreases as ATP is generated. Furthermore ATP generation is prevented by adding fluoride which inhibits enolase, the enzyme likely to be involved in the utilization of PGA as an energy source (Setlow & Kornberg, 1970a). More recent work by Setlow et al. (1977) shows that after the PGA is converted to pyruvate by enolase and pyruvate Kinase the pyruvate is further metabolized to acetyl CoA via pyruvate dehydrogenase with the production of NADH. Therefore the PGA supplies ATP, acetyl CoA and NADH, which are absent from dormant spores (Setlow & Setlow, 1977). The levels of acetate and NADH produced

Table 1

Comparison of the levels of some compounds found in dormant spores and vegetative cells of B. megaterium

Compound	Amount n mol/g wet wt.	
	Dormant Spores	Vegetative cells
ATP	3	725
ADP	94	-
AMP	450	-
CTP, GTP, UTP	< 10	680
cytidine nucleotides	133	150
guanosine nucleotides	107	310
uridine nucleotides	290	405
phosphoglyceric acid	6800	-

Data from Setlow (1975a)

Abbreviations: ATP, adenosine tri phosphate ADP, adenosine di phosphate AMP, adenosine monophosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

by germinating spores cannot be accounted for solely by the utilization of PGA and the deficiency may be made up from the metabolic action of amino-acid dehydrogenases, e.g. alanine dehydrogenase, on amino-acids produced from the extensive and selective protein degradation which occurs during germination (Setlow & Setlow, 1977; Setlow et al., 1977). This endogenous metabolism occurs in the absence of de novo protein synthesis, therefore all the necessary enzymes are present in the dormant spore.

Later during germination and outgrowth, when all the endogenous energy and nucleotide reserves have been utilized, normal metabolism takes over. Although the pathways have not yet been determined completely glucose is metabolized through glycolysis (EMP) and the hexose monophosphate shunt (HMP); measurements have been made of recoverable $^{14}CO_2$ from [1 - ^{14}C] - glucose and [6 - ^{14}C] - glucose during early and late outgrowth. In early outgrowth 80% was metabolized through the EMP and 20% through the HMP, later 90% by the EMP and 10% by the HMP, whereas vegetative cells metabolize 98% by the EMP and only 2% by the HMP (Blumenthal, 1965). There is therefore a greater contribution from the HMP during outgrowth. Similar results were obtained by Maruyama et al. (1980), who also reported that about 10-30% glucose was oxidized to gluconate, presumably by glucose dehydrogenase. The activity of this enzyme is high in spores (Warren, 1968), and it is possible that if glucose is oxidized directly to gluconate by $NADP^+$, the NADPH could be reoxidized by an electron transport system thus conserving energy by producing ATP without glucose phosphorylation (Maruyama et al., 1980). Also in early outgrowth there is little or no activity of the tricarboxylic acid cycle (TCA) (Setlow & Kornberg, 1970a), and the HMP affords the outgrowing spore a means for totally utilizing glucose independently of the TCA cycle. Respiration via membrane-associated electron transport is activated during germination of spores even in the

absence of protein synthesis (Wilkinson et al., 1977), although development of the cytochrome system during outgrowth has also been observed (Tochikubo, 1971).

There are therefore two main stages of energy metabolism during germination and outgrowth as described by Setlow (1975a). Stage I represents a turnover stage in which energy and energy-related functions are derived from endogenous reserves; during Stage II spores can synthesize all the required small molecules and have exhausted the reserves utilized in Stage I, during Stage II most of the biosynthetic processes of outgrowth occur. These are described in later sections (2.2 to 2.6).

2.2 RNA biosynthesis

RNA species are the first major macromolecules to be synthesized after germination of the spore and much of the energy produced by the spore is used in this synthesis. RNA synthesis can be supported for a short time in the absence of nutrients by utilization of ATP produced from PGA, amino-acids and nucleotides stored in the dormant spore (Setlow & Kornberg, 1970b). The nucleotides were found as free nucleotides although about 5% of the total spore RNA is degraded early in germination. The nature of the RNA species degraded is unknown but less than 5% of the tRNA species is degraded. (Setlow *et al.*, 1974) and it has been suggested by Setlow (1975a) that the mRNA identified in spores by Jeng and Doi (1974), or defective tRNA, may be involved. The spore mRNA's are not translated during outgrowth (Kobayashi *et al.*, 1965). The supply of nucleotides for RNA synthesis is sufficient for the RNA, synthesis during early outgrowth, in the absence of nucleotide synthesis during this period. Nucleotide synthesis as measured by incorporation of labelled aspartate into UTP, is absent until Stage II of energy metabolism and after this most of the UTP is produced *de novo*. The enzymes for this biosynthesis are not present in the dormant spore (Setlow & Kornberg, 1970b).

Early studies on transcription during outgrowth of *B. subtilis* reported that it was biphasic; in the early phase only rRNA was formed; later both rRNA and mRNA were synthesized, this stage was concurrent with the initiation of protein biosynthesis (Armstrong & Sueoka, 1968; Woese & Bleyman, 1969). On the other hand Balassa and Contesse (1965) reported that both rRNA and mRNA are synthesized from the beginning of *B. subtilis* spore outgrowth. Also in *B. cereus*, synthesis of all classes

of RNA species was reported by Spiegelman et al. (1969) and Torriani et al. (1969). A more sensitive probe of the nature of transcription during outgrowth that involving hybridization of the transcripts to the heavy (H) or light (L) strands of DNA, was used by Margulies et al. (1978). They have shown a decrease in the asymmetry ratio of RNA hybridized to the H and L strands during outgrowth and that about 50% of RNA synthesized during early outgrowth was ribosomal, decreasing to 35% later. At no time was rRNA the sole form synthesized. There was, however, a very large increase in the rate of rRNA synthesis prior to the onset of DNA synthesis. Sloma and Smith (1979) found a similar pattern. Early RNA synthesis was at a low level which increased rapidly, probably at germ cell emergence. From the results of following the specific activity of the UTP pool using [^{3}H] - uridine and from pulse labelling studies they suggested that the biphasic rate of RNA synthesis could be accounted for in two ways. First, as an increase in the permeability of the spore at the time of rapid synthesis, evidenced by the increase in the specific activity of the UTP pool. Secondly, by correcting for the increase in available label they showed that the actual rate of RNA synthesis increased. Hybridization competition studies with rRNA showed rRNA was synthesized throughout outgrowth. The UTP pool labelled during sporulation with [^{14}C] - uracil did not increase during outgrowth so it is unlikely that pre-existing spore RNA was degraded and the nucleotides re-utilized to give an apparent low rate of transcription early in outgrowth.

Armstrong and Sueoka (1968) and Donnellan et al. (1965) have shown that tRNA is synthesized continuously during outgrowth. These studies have given no insight into which species of tRNA's are synthesized and if they differ from sporulating or vegetative species. Recently a

study of tRNA species synthesized during vegetative growth, sporulation and outgrowth by two-dimensional polyacrylamide gel electrophoresis (PAGE) of ^{32}P-labelled RNA, has shown that there are no detectable differences in the species synthesized or their abundances during any of the stages of growth (Henner & Steinberg, 1979).

In summary, the outgrowing spore synthesizes all of the major RNA species, rRNA, tRNA and mRNA. The overall rate of synthesis is biphasic, this may be the result of a limitation of energy or of nucleotides during the early stages or of low levels of proteins involved directly in transcription e.g. DNA-dependent RNA polymerase. Clivio et al. (1979) have suggested that the rate of RNA synthesis during outgrowth is dependent on the amount of DNA present during outgrowth. In a later section the possible transcription controls operating are discussed.

2.3 Protein biosynthesis

Protein synthesis during outgrowth starts after RNA synthesis, and can be supported solely from endogenous reserves, at least in the early stages. This endogenous synthesis is supported from two sources; first, in B. megaterium, three amino-acids are found free in dormant spores; secondly, and more importantly, during germination large amounts of a further 15 amino-acids are found (Nelson & Kornberg, 1970; Setlow & Primus, 1975). These amino-acids are derived from spore proteins and Setlow (1975b) has identified three small-spore-specific proteins which account for 80% of the protein degraded during germination (Setlow, 1975c). The endoprotease that degrades these proteins is also found only in the spore and is specific for these proteins, the specificity appears to be due to the endoprotease recognising a particular amino-acid sequence(Setlow et al., 1980). Setlow (1976) has shown that these proteins are found in the core and may bind to DNA. Apart from supplying a source of amino-acids for protein synthesis during outgrowth they also provide some of the energy required (see Section 2.1). These proteins are important in the initiation of outgrowth because the dormant spore does not contain some of the enzymes necessary for amino-acid synthesis, these appear de novo during outgrowth just prior to the biosynthesis of the amino-acids themselves (Setlow & Primus, 1975).

Protein synthesis is essential for development of the outgrowing spore, inhibitors of protein synthesis prevent many of the processes which occur during outgrowth (Keynan, 1973). Protein synthesis itself is dependent on prior mRNA synthesis, since actinomycin D, an inhibitor of mRNA synthesis prevents protein synthesis (Steinberg & Halvorson, 1968), although it should be noted that actinomycin D has toxic effects apparently

unrelated to its ability to prevent mRNA synthesis (Korn et al., 1965; Laszlo et al., 1966) and these may influence results obtained using this drug. Nevertheless dormant spores are almost devoid of mRNA (see Section 2.2) therefore synthesis of mRNA is required before proteins can be synthesized. It therefore seems unlikely that reports that spores do not contain all the requirements for translation or that the system is defective can be correct (Kobayashi et al., 1965) rather the system is activated during germination or the methods used to isolate the in vitro systems are suspect. Recent evidence indicates that the latter alternative is the more likely (Keras et al., 1978).

One particular interesting feature of protein synthesis during outgrowth is its sequential nature. At different stages of outgrowth there are distinctive patterns of protein synthesis, and enzymes have been shown to be synthesized in a periodic manner throughout outgrowth (Kobayashi et al., 1965; Torriani & Levinthal, 1967; Steinberg & Halvorson, 1968; Yeh & Steinberg, 1978). It has been implied from these studies that cells control protein synthesis during outgrowth such that particular proteins are only synthesized at particular stages of outgrowth. This phenomenon is discussed in more detail later (Section 3.1). Unfortunately detailed two-dimensional PAGE analysis of the sequence of proteins synthesized at different stages of development, available for sporulating B. subtilis and Saccharomyces cerevisiae are not yet available for spore outgrowth (Linn & Losick, 1976; Wright & Dawes, 1979).

2.4 DNA Synthesis

Unlike RNA and protein synthesis, net DNA synthesis does not begin until late in outgrowth (Strange & Hunter, 1969). DNA synthesis appears to be unnecessary for most of outgrowth, since germinated spores can elongate and synthesize macromolecules and membrane lipids in the absence of DNA synthesis (Dawes & Halvorson, 1972; Ginsberg & Keynan, 1978), although there may be a decrease in the rate of RNA synthesis (Clivio et al., 1979). Dormant spores contain all of the enzymes needed to form deoxyribonucleotide triphosphates (d NTP's), but the nucleotides themselves are absent. All four appear early in outgrowth, generated by the reduction of ribonucleotides produced by the hydrolysis of RNA (see Section 2.2, Setlow, 1973a). The relative concentrations of dNTPs remain low until the onset of DNA synthesis, when they increase to about 10% of vegetative levels. This burst of synthesis has been attributed to increased levels of ribonucleotide reductase and it has been suggested that the levels of dNTP's may control this and the rate of DNA synthesis during outgrowth (Setlow, 1973a). Moreover, the replication enzyme DNA polymerase III, which is not detectable in spores, is synthesized just before the onset of DNA replication (Ciarrocchi et al., 1977). More recently DNA gyrase activity has been implicated in the initiation of DNA synthesis and elongation during spore outgrowth, although it is not clear whether the DNA gyrase acts directly on initiation and elongation or indirectly through RNA synthesis (Ogasawara et al., 1979). It seems likely that a combination of some or all of these features is responsible for the late initiation of DNA synthesis during outgrowth.

Sporulation in Bacillus spp can only be induced during a short

stage in the cell division cycle dependent on DNA replication (Dawes et al., 1971; Mandelstam & Higgs, 1974). Germination and outgrowth, on the other hand can apparently proceed normally in the absence of detectable DNA synthesis (Ginsberg & Keynan, 1978). Nevertheless DNA replication is one of the important regulated events in the cell cycle and is of considerable interest.

Dormant spores of B. subtilis contain diffuse, fully replicated chromosomes detached from the membrane (Yoshikawa & Sueoka, 1963; Oishi et al., 1964; Ryter, 1968; Callister & Wake, 1974). Reports that during sporulation thymine auxotrophs of B. subtilis produce forked chromosome structures, consistent with an incomplete round of chromosome replication (Ephrati-Elizur & Borenstein, 1971; Gillin & Ganesan, 1975) are probably the result of non-isogenicity between strains (Callister & Wake, 1974). The number of chromosomes present in dormant spores varies with species (Fitz-James & Young, 1959). B. subtilis has one chromosome whereas some B. megaterium spores have two chromosomes others only one, such that populations of spores are heterogeneous (Goldring & Wake, 1968; Wake, 1980).

Net DNA synthesis in outgrowing spores starts concurrently with germ-cell emergence and after RNA and protein synthesis have started (Steinberg & Halvorson, 1968; Lammi & Vary, 1972). Replication begins at the origin, continues towards the terminus and is bidirectional and symmetrical in both directions (Oishi et al., 1964; Gyurasits & Wake, 1973; Wake, 1974). DNA synthesis becomes resistant to chloramphenicol during outgrowth and it is likely that the enzymes necessary for this synthesis are not present in spores (Yoshikawa, 1965) or are degraded during outgrowth (Rana & Halvorson, 1972b).

Depending on the method used to measure DNA during outgrowth, two different patterns of synthesis are seen. Chemical analysis shows no increase in DNA during early outgrowth but by measuring the incorporation of [^{3}H] - thymine (or thymidine) into DNA a slight increase in incorporation is seen at about the time RNA synthesis begins followed by a lag period and then a final increase in the rate of labelling of DNA associated with chromosome replication (Rana & Halvorson, 1972b).

This initial low level of DNA synthesis has been the subject of several investigations but no clear picture of its significance has yet emerged. Yoshikawa (1965) found that the addition of chloramphenicol to outgrowing B. subtilis spores did not inhibit this incorporation, indicating that the spore contains enzymes capable of incorporating bases into DNA. Cs Cl density gradient analysis of DNA synthesized in the presence of chloramphenicol and bromodeoxyuridine (BudR), revealed that the DNA was of normal density and that the genetic marker frequency was consistent with that of non-replicating DNA. He concluded that the chloramphenicol-resistant DNA synthesis was probably an excision repair mechanism. A similar study by Wake (1967) showed that most of the initial uptake of label by outgrowing B. subtilis spores was probably associated with the presence of radioactive impurities in the label and also due to asynchronous initiation of DNA replication in the spore population. He calculated that any repair synthesis would be at an undetectable level. Repair-like synthesis in B. cereus T was similar to that found in B. subtilis by Yoshikawa (1965). That is, label was initially incorporated into DNA strands in a manner suggestive of excision repair; unlike B. subtilis this synthesis was prevented by chloramphenicol (Rana & Halvorson, 1972b). The early incorporation of label into DNA during outgrowth of B. megaterium spores was also sensitive to chloramphenicol and 6 - (p-hydroxyphenylazo)uracil, a specific

inhibitor of replicative DNA synthesis, thus it was concluded that there is little if any repair synthesis during outgrowth of B. megaterium spores (Lammi & Vary, 1972). The difference in chloramphenicol resistance of early DNA replication observed in B. subtilis and B. cereus T may be due to the extensive protein turnover during outgrowth in B. cereus that is lacking in B. subtilis (Rana & Halvorson, 1972b).

The conflicting evidence concerning repair synthesis during spore outgrowth has yet to be resolved. It is, however, clear that spores do contain the enzymes necessary for repair of damaged DNA (Fujita & Komano, 1975; Ciarrocchi et al., 1977). If this early DNA synthesis is due to necessary repair of DNA damaged during sporulation or an extended period of dormancy, spores of mutants unable to repair certain types of DNA damage would not be able to outgrow normally. Ultra-violet (U V) - sensitive strains of B. subtilis defective in both dimer cleavage and excision repair can complete outgrowth normally (Munakata & Rupert, 1972), as can DNA polymerase I - deficient mutants, although these cannot repair UV - induced damage since they were unable to fill gaps left after excision of photoproducts (Fujita & Kamano, 1975). Yeh and Steinberg (1978) have also shown that spores of B. subtilis strains sensitive to mitomycin C and UV show normal patterns of protein synthesis during outgrowth. Circumstantial evidence for the existence of, or requirement for, repair function during sporulation at least has been presented by Honjo et al. (1976) and by Ciarrocchi et al. (1977), who have shown that while the overall levels of DNA polymerase III drop during sporulation DNA polymerase I is synthesized and reaches a higher level than that found in vegetative cells of the two enzymes, DNA polymerase I activity alone is detectable in dormant spores. These results may indicate that repair of DNA is

required during sporulation or for outgrowth under certain conditions where there has been damage to the DNA during dormancy or germination. Overall there is no evidence that repair of DNA is essential for outgrowth.

There is some speculation as to whether the initiation of DNA replication during outgrowth is different from that occurring during normal vegetative replication, based on two observations. First, a mutant of B. subtilis temperature sensitive (ts) for initiation of DNA replication does synthesize some DNA at the restrictive temperature (45°C) during outgrowth (Mendelson, 1968); secondly, Galizzi et al. (1978) have isolated a mutant that is ts for DNA synthesis during outgrowth but not vegetative growth. Callister et al. (1977) have investigated the first ts mutant and confirmed that during outgrowth a significant proportion of the spore population does initiate replication. This could mean that the initiation sequence during outgrowth was primed during sporulation (it should be remembered that spores contain completed chromosomes) but they found that at higher temperatures, up to 49°C, the initiation was prevented. Therefore it seems more likely that the ts protein is not fully inactivated at 45°C in outgrowing spores, which may reflect differences in its environment in the spore compared with that found in vegetative cells (Callister et al., 1977). The ts mutant of Galizzi et al. (1978) could also be explained by the difference in internal environment of the spore, e.g. it may be that more stringent ion requirements of the ts product are not met in the spore and so initiation is not activated, these requirements being more easily met in a vegetative cell. Certainly DNA synthesis is more sensitive to specific inhibitors of DNA synthesis during outgrowth (Ginsberg & Keynan, 1978).

One interesting feature related to DNA synthesis during outgrowth

is the release of transforming DNA segments in a sequential genetic order (Borenstein & Ephrati-Elizur, 1969). This phenomenon has been investigated further by Orrego et al. (1978), who found these genetic markers to be resistant to DNase and tight physical contact between the spores and competent cells was required for transformation. The significance of this release of DNA is unknown at present. On the basis of the fact that outgrowing spores are themselves competent, coupled with the release of this genetic material during outgrowth, these authors have postulated that this may be some kind of primitive conjugation mechanism.

2.5 Cell wall synthesis

During germination of spores the bulk of the spore cortical peptidoglycan is autolysed but the germ-cell of the spore is not lysed by the osmotic shock caused by the hydration of the spore. It has been suggested that the innermost layer of the spore cortex may become the new cell-wall, so protecting the germ-cell from osmotic lysis. Electron micrographs of spores from a wide range of Bacillus species have shown beneath the cortex a thin, dark band which was susceptible to lysozyme degradation (Warth et al., 1963). Tipper and Gauthier (1972) have further suggested that during germination of spores, the cortical bilayer is subject to autolytic enzymes which degrade the main bulk of cortex peptidoglycan leaving this thin layer of lysozyme-sensitive peptidoglycan, which is resistant to spore autolysins, as osmotic protection. This autolysin-resistant layer may also function as a primer for vegetative cell wall synthesis.

There is evidence to support the above hypothesis from a study of cell wall synthesis by Vinter (1965). He labelled cells of B. cereus with [^{14}C] - diaminopimelic acid (DAP) during the formation of prespores and the maturation stages of sporulation. On resuspending these spores in an outgrowth medium those spores labelled during prespore formation retained [^{14}C] DAP-containing material, whereas those labelled during the maturation stage formed the autolysed fraction of cortical peptidoglycan. The [^{14}C] DAP-containing material retained during outgrowth was augmented by the uptake of low-molecular weight DAP-containing material. This was confirmed by the use of penicillin, which prevents polymerization of cell wall materials and cycloserine, which prevents the interconversion of L-alanine and D-alanine.

More recently studies with a strain of B. megaterium auxotrophic for DAP and lysine have extended the work of Vinter, (Cleaveland & Gilvarg, 1975). The use of this particular double mutant gave a spore population with peptidoglycan exclusively labelled with radioactive DAP and also allowed independent control of protein and cell wall synthesis. During germination in a medium not able to support outgrowth 20% of radioactive DAP was retained independently of any protein or cell wall synthesis. When germination and outgrowth were followed in rich medium 20% of the cortex was still retained and transferred to outgrowing cells, although the rate of release was faster in rich medium. The degree of cross-linking in dormant spore and germinated spore peptidoglycan was 17% and 42% respectively and this 42% cross-linked fraction was present throughout germination. These results confirmed the earlier work by Vinter (1965) that only part of the cortical peptidoglycan is solubilized during germination. The difference in degree of cross-linking between dormant and germinated spore cortex led the authors to suggest that the spore cortex is solubilized during germination by activating autolytic enzymes but that the highly cross-linked peptidoglycan is resistant to hydrolysis and becomes part of the outgrowing spore cell wall. To date there is no evidence to suggest that this cross-linked peptidoglycan is spore-specific (Cleaveland & Gilvarg, 1975). The timing of the appearance of enzymes needed to synthesize new cell wall material was studied by the addition of chloramphenicol (CM) to outgrowing spores of various times. A requirement for prior protein synthesis was demonstrated before [^{14}C] DAP was incorporated into new wall material; similarly addition of actinomycin D prevented cell wall synthesis. It was concluded from these results that the synthesis of cell wall during outgrowth required synthesis of new mRNA and protein and that synthesis of both continued throughout development

(Vinter, 1965).

The dormant spore contains no teichoic acid but teichoic acids are synthesized during outgrowth (Boylen & Ensign, 1968). In B. subtilis W23, which contains polyribitolphosphate (PRP) and glucosylpolyribitol phosphate (GPRP), both teichoic acids were synthesized soon after germination. Actinomycin D totally inhibited teichoic acid synthesis but chloramphenicol, puromycin and penicillin selectively prevented synthesis of PRP with only 50% inhibition of GPRP. It appears that de novo enzyme synthesis is not necessary for the synthesis of GPRP; the apparent conflicting results using the inhibitor actinomycin D may be due to the toxicity of this drug (see Section 2.3). There was some GPRP transferase activity in spore extracts but none of the enzymes required for polymerization of teichoic acids could be found. Germinated spores were able to synthesize the uridine diphosphate D-glucose and cytidine diphosphate ribitol donor molecules required for polymerization of teichoic acids. B. licheniformus teichoic acid synthesis also depended on prior enzyme synthesis (Chin et al., 1968).

2.6 Membrane synthesis

Dawes & Halvorson (1972) examined membrane lipid synthesis during outgrowth of B. cereus T spores by following the incorporation of [^{3}H] - glycerol into chloroform-methanol soluble material. They found that membrane synthesis was discontinuous; after germination there was a 50 min period of membrane synthesis followed by a period of no lipid synthesis. Concurrent with a net increase in DNA at 110 min, lipid synthesis resumed in a linear fashion. At 180 minutes there was a sudden increase in the incorporation rate coincident with septation. During the early period of outgrowth, from 60 to 120 minutes, there was little turnover of membrane, later in outgrowth the turnover rate increased to about 30%, therefore the observed differences in the kinetics of lipid synthesis represented the true pattern of membrane synthesis. Chloramphenicol added at different times during outgrowth showed that the enzymes involved in membrane synthesis were present in the spore and that for the first 110 minutes lipid incorporation was not coupled to protein synthesis. Directly on addition of chloramphenicol a fixed rate of synthesis was observed from 110 to 180 minutes. It seems likely that an enzyme present at 50 minutes was degraded or inactivated before re-synthesis or activation at 110 minutes. The third rise in the rate of membrane lipid synthesis, seen at septation, was chloramphenicol resistant, the authors suggested that at this time a regulatory protein for lipid incorporation or membrane synthesis was synthesized. In the absence of mRNA synthesis, achieved by adding actinomycin D, only the rapid incorporation of label into lipids was inhibited. Membrane synthesis occurred in the absence of DNA synthesis and cell septation, the same pattern of membrane synthesis was observed in the presence of nalidixic acid. The synthesis of regulatory factors involved in lipid biosynthesis, may also function during the synthesis of fatty acids

during outgrowth of B. thuringiensis, (Bulla et al., 1975). Table 2 shows the relative amounts of isomers, anteisomers and normal chain fatty acids synthesized from [1-^{14}C] - acetate during outgrowth.

Table 2

Relative specific activity of branched and normal chain fatty acids synthesized during outgrowth of B. thuringiensis spores

Outgrowth time (min)	ISOMERS	ANTEISOMERS	NORMAL
0-30	99.9	0	0
30-60	35.4	32.5	32.2
60-90	39.5	20.4	40.1
90-120	44.3	27.3	25.9

From Bulla et al. (1975)

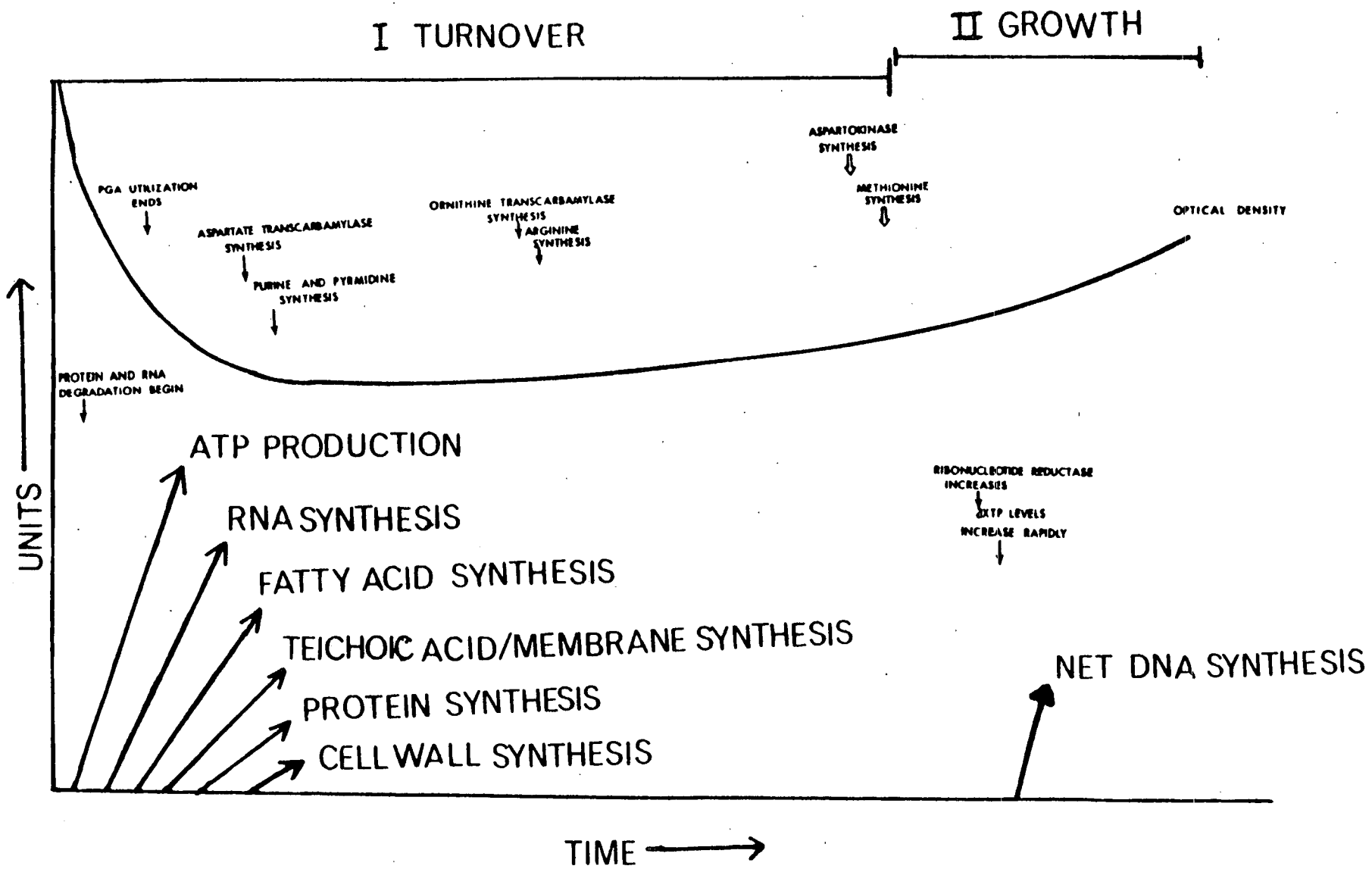
As can be seen there are two distinct stages of fatty acid synthesis during outgrowth: the first stage which appears in the absence of protein synthesis consists solely of isomer synthesis, possibly reflecting spore specific synthesis: the second stage beginning at 30 mins. is characterised by the synthesis of normal chain fatty acids. However, these second stage normal chain fatty acids have relative specific activities which far exceed their relative abundances in either spore or vegetative cell membrane. Also the relative specific activity of each individual fatty acid varies substantially through outgrowth. This

differential synthesis may indicate that regulatory mechanisms control membrane fatty acid composition and such changes determine the development of membrane functions during outgrowth (see Section 3.3, Bulla et al., 1975).

2.7 Summary

Before considering the control systems that operate during spore outgrowth it is worth summarising the biochemical aspects as a whole. The temporal relationships between the major events occurring during outgrowth are shown diagramaticaly in Figure 3 based on the review of Setlow (1975a). Stage I can be divided into three parts, a, b and c. All Stage I events listed are based on the metabolism of endogenous reserves. During stage Ia PGA is utilized to produce ATP, acetate and NADH; later RNA and protein is broken down, these reserves of nucleotides and amino-acids provide the building blocks and part of the energy for initiation of RNA, protein and DNA synthesis in Stage Ib. The spore at this time is unable to synthesize nucleotides and amino-acids *de novo*. This enables the outgrowing spore to reach Stage Ic, in which some mRNA is synthesized and translated to provide several enzymes, that can synthesize new amino-acids and nucleotides the cell can now progress to Stage II. All the activities in Stage I are supported by endogenous reserves and therefore there is no net biosynthesis of RNA, protein, DNA, cell wall etc. to allow elongation of the spore and eventually cell division. While much of the above is from studies of *B. megaterium*, Figure 3 is a composite picture including studies of *B. cereus*, *B. thuringiensis* and *B. subtilis* and as such only a general representation of the biochemistry of outgrowth.

Figure 3. Temporal relationships of main biochemical events during spore outgrowth. Based on a review by Setlow (1975a).


I TURNOVER
II GROWTH
PGA UTILIZATION ENDS
ASPARTATE TRANSCARBAMYLASE SYNTHESIS
PURINE AND PYRIMIDINE SYNTHESIS
ORNITHINE TRANSCARBAMYLASE SYNTHESIS
ARGININE SYNTHESIS
ASPARTOKINASE SYNTHESIS
METHIONINE SYNTHESIS
OPTICAL DENSITY
PROTEIN AND RNA DEGRADATION BEGIN
UNITS
ATP PRODUCTION
RNA SYNTHESIS
FATTY ACID SYNTHESIS
TEICHOIC ACID/MEMBRANE SYNTHESIS
PROTEIN SYNTHESIS
CELL WALL SYNTHESIS
RIBONUCLEOTIDE REDUCTASE INCREASES
dTP LEVELS INCREASE RAPIDLY
NET DNA SYNTHESIS
TIME

3. Control of spore outgrowth

When considering the biochemistry of outgrowing spores it is apparent that the process involves an ordered sequence of events, leading from a dormant structure to an actively dividing vegetative cell. Thus after the initiation of transcription a small number of proteins is synthesized followed by the sequential synthesis of more protein species to give an increasingly more disperse population of proteins (Kobayashi et al., 1965; Steinberg & Halvorson, 1968). When mRNA synthesis was blocked with actinomycin D the later events of outgrowth were prevented (Steinberg & Halvorson, 1968), and although results obtained using actinomycin D are open to alternative interpretations, there is other evidence that the initiation of these events is controlled at the level of transcription (see Section 3.3). The sequential changes during outgrowth can be observed in mRNA populations synthesized at various times during outgrowth (Spiegelman et al., 1969; Hansen et al., 1970). Previously the biochemical changes occurring during outgrowth have been considered in isolation without discussing how they are controlled or correlate with each other. The following sections are an attempt to build up a picture of this control of the morphogenetic process in as much detail as possible.

3.1 Ordered protein synthesis during spore outgrowth

The ordering of protein synthesis during outgrowth is most easily investigated by studying enzyme synthesis, since enzyme activities can be measured without having to use the elaborate controls necessary in immunoprecipitation techniques. Kennett & Sueoka (1971) have shown that several enzymes appear in a sequential manner during outgrowth. The enzymes, sucrase, trehalase, ornithine transcarbamylase, aspartate transcarbamylase and threonine dehydratase all appeared before the onset of DNA replication except for ornithine transcarbamylase. In *B. subtilis* mutations in the genes coding for these enzymes were mapped by density transfer techniques. By comparing the order in which the enzyme activities appeared during outgrowth with the position of the gene on the chromosome they found that the sequence of enzyme synthesis corresponded to the order of the related genes on the chromosome, although initially ornithine transcarbamylase did not appear. As DNA replication proceeded the enzymes were again synthesized in the same order and this time ornithine transcarbamylase appeared at the time expected from its position on the chromosome. Therefore it was considered that during outgrowth the chromosome was transcribed sequentially and this order was important in the normal development of the outgrowing spore. This hypothesis was supported in part by the work of Armstrong & Sueoka (1968) who suggested that RNA synthesis during outgrowth was biphasic, i.e. rRNA was synthesized first during early outgrowth followed by mRNA, because the genes coding for rRNA are near the origin of the chromosome (Smith *et al.*, 1968; Moran & Bott, 1979), this would be consistent with sequential transcription of the chromosome. Also some temperature-sensitive mutants blocked during outgrowth could be grouped on the basis of the stage at which development was blocked and this

related to the position of the mutation on the chromosome (Fig. 4, Nukushina & Ikeda, 1969).

When the above work was done the chromosome of B. subtilis was thought to be linear, but even with the rearrangement of the genetic map to the circular form there was still good correlation between the relative time of enzyme synthesis and gene position (Yeh & Steinberg, 1978). However, Yeh and Steinberg (1978) used B. subtilis strains with translocated segments in their chromosomes to investigate the necessity of gene order for the sequence of events demonstrated during outgrowth. Two enzymes were studied, trehalse (tre) an early enzyme and threonine deaminase (ilv A) a late enzyme. The position of tre was altered to the left or right of the origin and ilvA was altered with respect to position on the chromosome and copy number (Fig. 5). The results obtained showed that the timing and pattern of tre synthesis was not altered in strain GSY 1208, the translocated strain, when compared with GSY 266, the wild type. Furthermore, ilvA expression was unaltered in all three strains used, although in GSY 1961 (the translocated merodiploid) twice as much enzyme was produced, reflecting the gene dosage. The timing of aspartokinase synthesis in a mutant derepressed for this enzyme was also unaltered. Spiegelman et al.(1969) have also shown that an enzyme can only be induced during outgrowth when it is being synthesized. Yeh and Steinberg (1978) made several points from their study: gene position with respect to the origin does not determine the temporal sequence of enzyme synthesis; enzyme synthesis during outgrowth reflects gene dosage; and, altering the sequence of genes on the chromosome does not alter the sequence of events during outgrowth

The last statement is not fully substantiated, but there is other evidence which supports the hypothesis that the order of genes on the

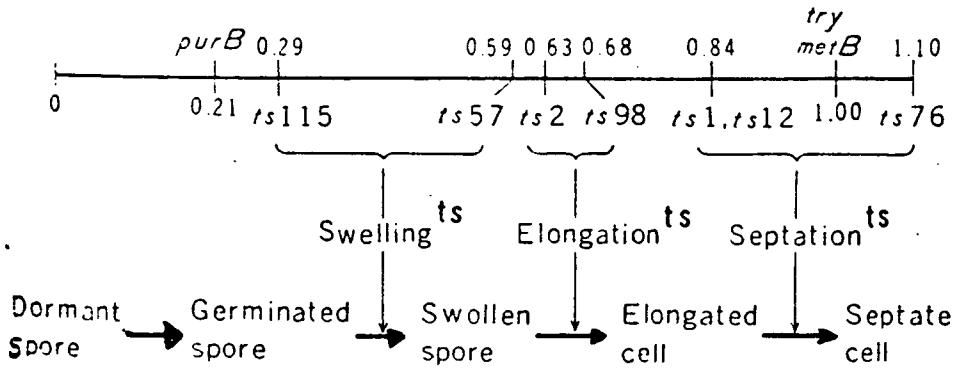


Figure 4. Relationship between location of temperature sensitive outgrowth mutations and order of expression of genetic lesion. From Nukushina and Ikeda (1969). Map distances were calculated from transformation marker frequency of ts markers to try. Standard abbreviations of genetic markers listed by Young and Wilson (1975) have been used.

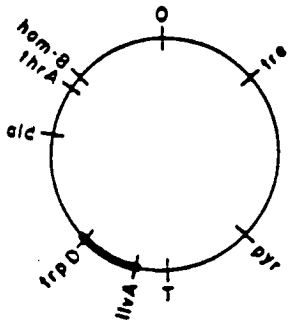
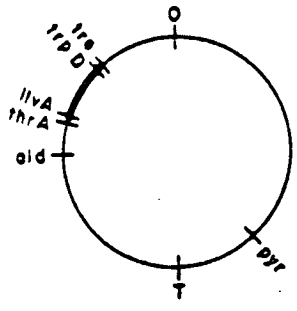
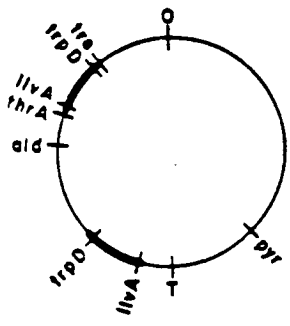
STRAIN		Time of synthesis (min)	
		Trehalase	Threonine deaminase
GSY 266		0,120,200	70,200
GSY 1208		0,120,200	50,200
GSY 1961			50,200 (twice the amount)

Figure 5. Time of expression of trehalase and threonine deaminase activity during outgrowth of strains of B. subtilis with translocated segments of chromosome. Data from Yeh and Steinberg (1978). Standard abbreviations of genetics markers listed by Young and Wilson (1975) have been used.

chromosome does not dictate the order of events occurring during outgrowth. Early in outgrowth mRNA cistrons are transcribed to about the same extent as rRNA ones (Margulies et al.,1978). The genes coding for rRNA are near the origin of the chromosome, interspaced with tRNA genes and 5s rRNA clusters (Smith et al., 1968; Moran & Bott, 1979). Therefore if the chromosome were transcribed from the origin there would be a period of relatively high rRNA synthesis compared to mRNA which is not the case (Margulies et al., 1978). More direct evidence has been provided by Silberstein and Cohen (1978), who hybridized RNA transcripts from outgrowing spores to endonuclease-treated DNA, and found that transcription was not limited to just one area of the genome but was distributed throughout it. The model that seems to best fit the data is selective transcription from genes at various positions and not sequential transcription from origin to terminus.

3.2 mRNA patterns during outgrowth

From the studies of protein biosynthesis a number of predictions can be made concerning the pattern of transcription during outgrowth. The mRNA population should be restricted to a few species early during outgrowth, become more heterogeneous as outgrowth proceeds and it should show the periodicity displayed by the enzymes, i.e. the individual messages fluctuate during outgrowth. Several investigations concerning mRNA populations synthesized during outgrowth have been made.

Hansen et al. (1970) in an RNA/DNA hybridization study, using B. cereus RNA, competed RNA isolated during outgrowth against pulse-labelled transcripts from outgrowing spores; rRNA was competed out. The data were presented as a series of double-reciprocal plots of the competition curves obtained and Table 3 shows the slope of these data, a low value represents similarity and vice versa. As could be predicted, there were only small differences between transcripts synthesized at similar times e.g. 8 minute RNA competed almost completely with 17 minute RNA. The periodicity of protein synthesis fits well with their data, there was little competition between 8 minute and 40 minute mRNA but between 8 minutes and 80 minutes there was extensive competition again, therefore the species synthesized at 8 minutes were not synthesized to any great extent at 40 min but were synthesized again at 80 min (Hansen et al., 1970). Any interference, in these data, from precurser rRNA synthesized during outgrowth (Sloma & Smith, 1979) would be minimal because of the short duration of the pulse and since rRNA is synthesized throughout outgrowth.

In a more detailed work in which vegetative cells were compared with outgrowing spores, Margulies et al. (1978) examined the asymmetric

Table 3

Hybridization competition analysis of mRNA populations during outgrowth at different times after germination (Hansen et al., 1970)

Time into outgrowth (minutes)		
Labelled RNA	Unlabelled	Slope*
8	17	6.0
8	40	Large
8	80	14
40	8	16
40	80	5.5
80	40	Large

A large slope represents dissimilarity.

* Slopes were calculated as: a plot of $1/(1-F)$ relative to C'/C; where F is the fraction of labelled RNA which is unaffected by the addition of unlabelled RNA, C' is the concentration of labelled RNA, and C is the concentration of unlabelled RNA.

transcription from the heavy (H) and light (L) strands of DNA by hybridization. The authors divided outgrowth into several stages, Stage 1 (30 min), Stage 2 (45 min), Stage 3 (90 min), and Stage 4 (160 min). There was a gradual decrease in the relative transcription from the H and L strands of B. subtilis DNA (H/L ratio) during outgrowth with the amount of L strand transcripts increasing until the levels approached those found in vegetative cells i.e. from an H/L of 7.5-9.7 during early outgrowth to H/L of 3.2-4.6 in vegetative cells. The amount of transcription from the H strand did not change significantly from stage 1-4, though there may have been a slight decrease from Stage 1 compared to vegetative growth. Although rRNA and tRNA are transcribed exclusively from the H strand (Margulies et al., 1978) the observed asymmetry was not due to a contribution from stable RNA because the ratios of RNA/DNA in the hybridizations were high, the level of rRNA genes being small over the genome compared to message regions.

The next obvious question is whether any of the mRNA transcripts are specific to outgrowth. Setoguchi et al. (1978) carried out hybridization-competition analyses to determine the extent of transcription that was specific to outgrowth, and the pattern of expression of log-phase genes during spore outgrowth. They found that outgrowth-specific genes were represented in transcripts from both H and L DNA strands, most were found in transcripts from the H strand. During Stage 1 of outgrowth 10-15% and 1-3% transcripts from the H and L strands respectively were unique, by Stage 2 this had fallen to 5 and 2% respectively which represented a 67% overall decrease in H strand transcripts but only 35% from the L strand. Stage 4 transcripts were completely competed out by vegetative RNA. The transcription from vegetative genes started in Stage 1 from both H and L strands (Setoguchi et al., 1978).

Although it appears that there are specific genes for outgrowth transcribed during the process the above study did not compete out sporulation mRNA so these outgrowth - specific transcripts may have been associated with sporulation. Indeed Doi (1965) found no specific mRNA in outgrowing spores when competing against sporulation mRNA and vice versa. Silberstein and Cohen (1978) visualized transcribed DNA from outgrowing B. cereus spores by means of autoradiography of endonuclease treated DNA hybridized with ^{32}P-labelled RNA. They competed vegetative RNA against outgrowth transcripts and showed that one region of the chromosome was not competed out, although its intensity decreased with increasing vegetative RNA. They concluded that there was not much if any specific mRNA transcription during outgrowth but at least one transcript is present in much greater amounts during outgrowth than in vegetative cells.

There is, however, some evidence of outgrowth-specific genes provided by the studies of Galizzi and his colleages, who have isolated mutants that are temperature-sensitive only for outgrowth. These are described below. To date genes for outgrowth have not been cloned by genetic manipulation; if they were isolated in this way they would prove invaluable for studying the transcription of individual messages during outgrowth and all other stages of the Bacillus life cycle.

3.3 Temperature-sensitive outgrowth mutants

The hypothesis that there exist specific genes controlling outgrowth at least at the level of transcription to mRNA is unproven, however if there are such genes it should be possible to isolate mutants that are temperature sensitive (ts) solely for outgrowth. One of the advantages in looking for such morphogenetic mutants in Bacillus species is that all the genes are conserved at every stage of the life cycle.

Nukushina and Ikeda (1969) have found that ts vegetative-growth mutants of B. subtilis were blocked during outgrowth at various morphologically recognizable stages and that the block correlated directly with the genetic order from the chromosome origin of the ts loci (see Section 3.1). Because these mutants were blocked during vegetative growth and indeed were not selected on the basis of the inability to outgrow they are not specifically ts-mutants of outgrowth. Dawes and Halvorson (1974) selected outgrowth mutants on the basis of the density changes which occur during outgrowth, thus they enriched for ts mutations by germinating a mutated spore population at 45°C and separating the spores on Urografin gradients. 54 independent mutants were isolated of which all were defective in vegetative functions at 45°C. From a screening of 100,000 colonies, 0.1% were asporogenous but none were affected solely in outgrowth. The authors concluded that there are very few if any genes unique to outgrowth. This may be based on a false premise (i.e. that each of the mutants had only one lesion) because the mutagenic agent used N-methyl-N-nitro-N-nitrosoquanidine, can cause multiple lesions. Therefore a more realistic appreciation of outgrowth specific genes would have been obtained if the mutation had been crossed back into the wild-type to reduce the possibility of multiple lesions.

Much of the work concerned with ts mutants of spore outgrowth has been done in the laboratory of Galizzi who to date has isolated 32 mutants ts in only outgrowth (Galizzi et al., 1973; Galizzi et al., 1975; Galizzi et al., 1978; Albertini et al., 1979). . These mutants fall into two-main classes: those altered morphologically; and those showing a defect in the synthesis of macromolecules (Table 4). The morphological mutants, gsp IV form large swollen cells following outgrowth at 47°C, they are all unaffected in macromolecular synthesis, and can be phenotypically cured by sucrose (20%), Na Cl (2%) and sublethal doses of distamycin A ($>10 ugml^{-1}$) suggesting that they may be affected in a membrane function (Siccardi et al., 1975). The spores do form the first septum, although it does not divide the cell into discrete compartments (Galizzi et al., 1978). It seems likely that these mutants have a lesion in a protein involved in cell envelope synthesis or membrane stability, and that this function is not required in either germinating spores, vegetative or sporulating cells.

The mutants affected in macromolecular synthesis are particularly pertinent to the study of outgrowth control. To date only one has been extensively characterised that is ts gsp 81, this mutant was blocked in RNA synthesis (Albertini & Galizzi, 1975). When outgrowing spores were incubated at 47°C RNA synthesis started at the same rate as the wild type but after 35 min the rate of synthesis fell to zero. The RNA transcripts produced at 47°C included both ribosomal and mRNA the mutant only synthesized six detectable polypeptides with molecular masses from 47-78K (Galizzi et al., 1976). Inhibiting the synthesis of protein at 47°C, resulted in an increase of RNA synthesis to almost the levels obtained at 35°C; however, the later during outgrowth protein synthesis

Table 4

Some characteristics of temperature-sensitive mutants of B. subtilis affected during outgrowth

				Macromolecular synthesis		
n	Phenotype	Time of block (min)	Phenotypic repair by sucrose/NaClor distamycin	RNA	Protein	DNA
8	tsGspIVa4	60-90	Yes	Not done	Normal	Normal
6	tsGspIVb1	60-90	Yes	Not done	Normal	Normal
0	tsGspIVc1	60-90	Yes	Not done	Normal	Normal
4	tsGspIV11	60-90	Yes	Normal	Normal	Normal
5	tsGspIV2	60-90	Yes	Normal	Normal	Normal
9	tsGspIV24	60-90	Yes	Normal	Normal	Normal
2	tsGsp4	20	Not done	Reduced	Reduced	None
8	tsGsp1	30-60	Not done	Not done	Reduced	Reduced
7	tsGsp81	40	Yes	Reduced	Reduced	None
9	tsGsp42	45	Not done	Normal	Reduced	None
0	tsGsp25	60	Not done	Normal	Normal	Reduced

from Galizzi et al., 1973; Galizzi et al., 1975; Albertini & Galizzi, 1975; tini et al., 1979.

was prevented, the less transcription was stimulated. The populations of RNA present in samples incubated in the presence and absence of protein synthesis, at 47°C, were different, therefore the effect was not just one of increased transcription of the same RNA species (Galizzi et al., 1976). Two possible explanations of the data were given: either a negative control repressor protein binds irreversibly to DNA at 47°C, and is not synthesized when protein synthesis is prevented, so allowing transcription from a wider range of genes: or a positive control operates, one involving an RNA polymerase subunit which interacts with the enzyme reducing the efficiency of transcription at 47°C. Preventing synthesis of this subunit allows "normal" transcription. (Albertini & Galizzi, 1975). Purified RNA polymerase from tsgsp 81, however did not show in vitro temperature sensitivity (Galizzi et al., 1978).

The gsp mutants map in four areas of the genome (Fig. 6) all the gsp VI mutants map in a single area, whereas those affected in macromolecular synthesis map in different areas. There is no evident correlation between the timing of each block and its position on the genome, unlike the results found by Nukushina and Ikeda (1969). Further characterization of all the ts mutants is required before one can deduce more about outgrowth control from them. The fact that there are mutations only ts during outgrowth is not proof that there are genes specific to outgrowth. They may just reflect the different environment present in outgrowing spores, some indication of this was given earlier in section 2.4 with the finding that a ts DNA initiation mutant showed a more stringent temperature requirement to inactivate the ts product. However, this does not detract from the usefulness of specific ts mutants affected in outgrowth for studying control during outgrowth, clearly

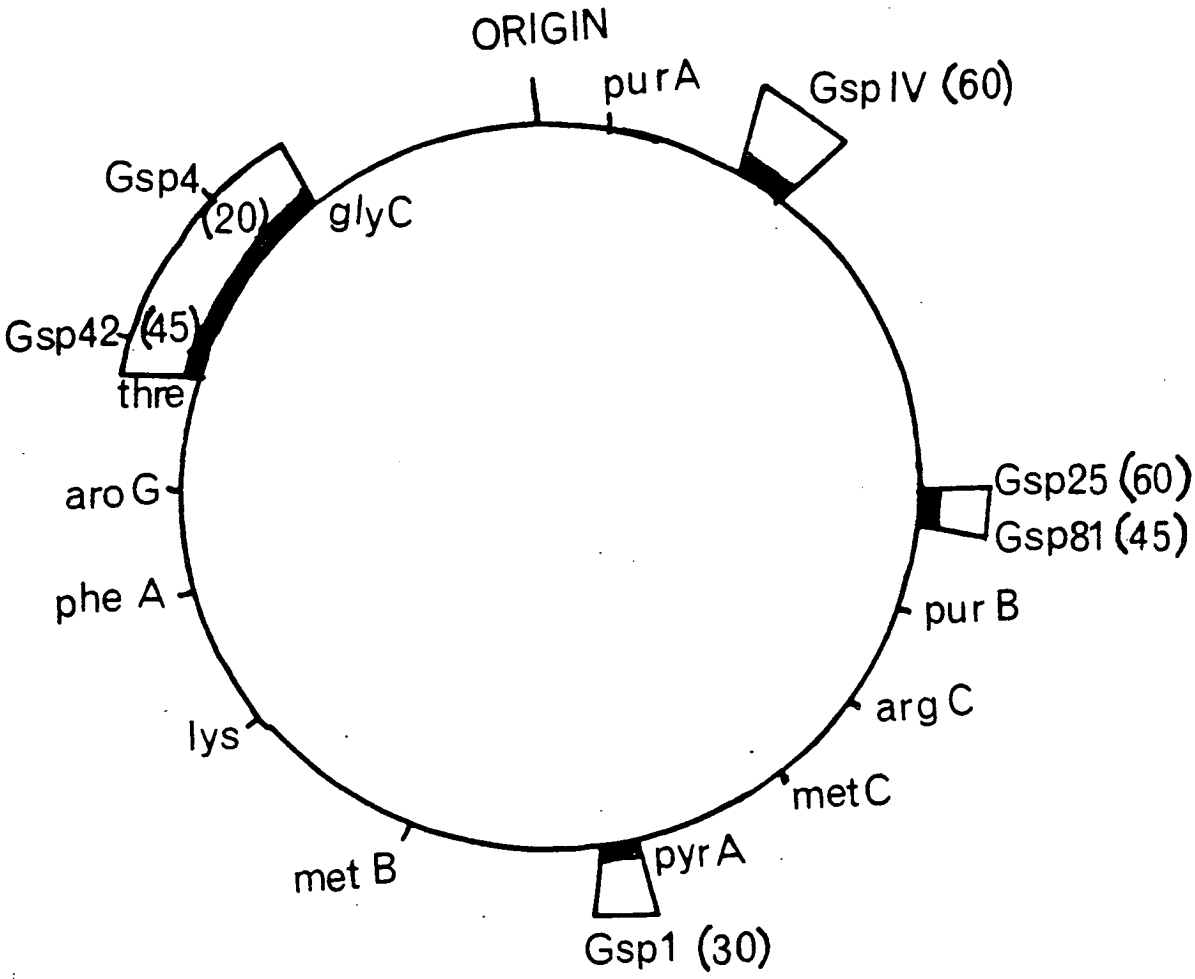


Figure 6. Location of gsp mutations on the B. subtilis genetic map. From Albertini et al.(1979). Numbers in brackets reflect the time of the ts block in minutes. Standard abbreviations of genetic markers listed by Young and Wilson (1975) have been used.

there are several genes which are controlled in a different manner or have products with more exacting requirements during outgrowth than in vegetative or sporulating cells. Therefore analysis of these ts mutants should shed some light on the regulation of outgrowth.

4.0 Transcriptional control during outgrowth

4.1 RNA polymerase specificity

We have seen that outgrowth may be controlled at the level of transcription, and it is well known that DNA-dependent RNA polymerase is involved in the control of transcription during sporulation, mediated by changes in sigma factor (σ) (Sonenshein & Campbell, 1978). Is there any evidence for this form of control operating during spore outgrowth? The specificity of spore RNA polymerase is similar to that found in sporulating cells, Ø e DNA is transcribed poorly by early outgrowth RNA polymerase (Ø e DNA can be considered to simulate vegetative promoters) and the enzyme contains little bound σ factor (Buu & Sonenshein, 1975; Linn et al., 1973). Later during outgrowth, σ factor activity is partially restored allowing expression of Ø e DNA. Table 5 shows some of the results of these experiments. Dormant spore and phase dark spore RNA polymerase read Ø e DNA at a low level (dark spores are in Stage I metabolism, see Section 2.7) but poly (dA-dT), which does not require σ factor for transcription, was read normally. After 40 minutes of outgrowth there was a large increase in Ø e transcription which remained almost constant throughout outgrowth, vegetative levels of ØeDNA transcription were not reached during outgrowth.

The sedimentation characteristics of RNA polymerase isolated from dormant spores differed from those from outgrowing spores and vegetative cells, and this behaviour was consistent with the spore enzyme lacking bound σ factor. Sigma factor activity was present in all extracts after zonalcentrifugation, although it was not bound to the enzymein

extracts from dormant spores. It was concluded that both RNA polymerase and σ factor were present in the dormant spore, but during germination and early outgrowth σ factor was prevented from binding to core RNA polymerase by a third component. It was suggested that this component was separated from the σ factor or enzyme by the density-gradient centrifugation. During outgrowth the inhibitor is either inactivated or degraded so restoring normal σ factor activity during outgrowth (Buu & Sonenshein, 1975). There is no direct evidence that such a component exists but if it does it may also operate during sporulation (Segall et al., 1974).

Further evidence that may show that RNA polymerase changes its specificity in outgrowing B. cereus spores has been provided by Cohen et al. (1973) and Cohen et al. (1975). When bacteriophage CP51 was incorporated into spores and these spores subsequently grown out, transcription of CP51 DNA was supressed during early outgrowth and developed only later. Addition of chloramphenicol to prevent protein synthesis did not prevent transcription of CP51 DNA, but led to selective transcription of the phage during early outgrowth (Cohen et al., 1973). A similar effect was shown in spores germinated in a medium unable to support outgrowth, histidase activity appeared earlier during outgrowth under these conditions, this behaviour is reminiscent of that shown by the ts mutant gsp81 described earlier. Earlier work by Cohen and Keynan (1970) with B. cereus had shown that protein sysnthesis was required for the stimulation of RNA synthesis observed in outgrowing spores, furthermore, the protein (proteins) that is synthesized early, may determine the pattern of proteins synthesized (Mazor et al., 1974). It therefore appears that there is a factor which suppresses transcription of some genes synthesized early in

Table 5

Template specificity of RNA polymerase from B. subtilis spores during outgrowth

Stage of outgrowth	Ratio of activity [øDNA/poly(dA-dt)n] Ammonium sulphate enzyme*
Refractile spores	0.11
Dark spores	0.15
Outgrowth:	
10 min	0.16
40 min	0.27
120 min	0.22
Vegetative cells	0.62

Data from Buu & Sonenstein (1975)

* Ammonium sulphate precipitated extracts of outgrowing spores and vegetative cells.

outgrowth and which increase transciption of others. By preventing synthesis of this protein some genes are transcribed earlier than normal. It is possible that these early suppressor/stimulating proteins could be new σ factor(s). Although this may just represent the return to the vegetative RNA polymerase specificity from the sporulation-specific reading shown in B. subtilis (Haldenwang & Losick, 1979) in reponse to changing nutrient conditions. It has been shown that under certain nutrient conditions outgrowing spores can resporulate (in a process termed microcycle sporulation), therefore the development of the cell is affected by nutrient status during outgrowth (Vinter & Slepecky, 1965). Transcription during early outgrowth may consist of a large amount from sporulation-specific genes, including sporulation σ factors and less transcription from vegetative genes. If the sporulation σ factor had a greater affinity for the core polymerase under the conditions of early outgrowth, failure to synthesize sporulation σ factor would lead to relatively more transcription from vegetative genes at an early time during outgrowth (this would include in vivo transcription from Ø e DNA). The observed asymmetry of transcription from H and L strand DNA, which changes during outgrowth may indicate a change in the class of promoters read by RNA polymerase (Margulies et al., 1978). Transcription from L strand DNA is higher in both outgrowing and sporulating cells than vegetative cells (Setoguchi et al., 1978).

It would be interesting to test the specificity of the "outgrowth" RNA polymerase with the plasmid of Haldenwang and Losick (1979) which contains genes under both vegetative and sporulation σ factor control; this would provide a sensitive probe of the relationships between the "outgrowth" RNA polymerase(s) and either the vegetative form of the enzyme

on the one hand or the late sporulation form on the other.

4.2 DNA-Gyrase activity

Recent studies indicate that DNA-gyrase, an enzyme which inserts negative superhelical twists into DNA, may be involved in the selection of genes (Smith et al., 1978; Sanzey 1979). Gottfried et al. (1979) have shown that novobiocin, which is thought to be a specific inhibitor of DNA-gyrase, preferentially inhibits outgrowth of spores, and this has led them to suggest that there is a special requirement for DNA-gyrase activity during outgrowth, these results however, may be the result of differential uptake of the antibiotic (see chapter 6). Nevertheless chromosomes in dormant spores of B. subtilis are diffuse and detached from the membrane; when spores outgrow, the chromosome condenses and binds to the membrane (Ryter, 1968; Ryter et al., 1968). Matsuda and Kameyama (1980) have proposed DNA-gyrase as a likely topoisomeric enzyme to cause this condensation. They have shown that there is a period of novobiocin-resistant transcription early in outgrowth which later became sensitive to the drug. There are two explanations for these data: either there is an increasing fraction of genes whose transcription is sensitive to novobiocin as outgrowth proceeds, therefore demonstrating a requirement for DNA-gyrase activity in the sequential regulation of gene expression: or the spore is less permeable to the drug very early during outgrowth. The latter explanation seems more likely as increased levels of novobiocin added early during outgrowth inhibited some of this novobiocin "resistant" fraction of transcripts (Matsuda & Kameyama, 1980). It is therefore too early to say if DNA-gyrase has any special involvement in controlling transcription during outgrowth.

4.3 Small molecules

There has been much speculation about the role of small molecules in control or initiation of sporulation. For example the levels of highly-phosphorylated nucleotides are closely linked with the ability of the cell to sporulate, in particular adenosine 3"5" bistriphosphate (P_3AP_3), and early sporulation mutants (SpoOF) did not produce P_3AP_3 or synthesize sporulation-associated enzymes such as alkaline phosphatase (Rhaese et al., 1978). However in mutants of B. subtilis which have lost the ability to control the levels of these nucleoticles i.e. rel mutants, the cells can still sporulate (Nishino et al., 1979). A similar situation exists with highly-phosphorylated guanine nucleotides e.g. pppGpp and ppGpp, and it is unlikely that any of the highly-phosphorylated nucleotides are involved in the initiation of differentiation in Bacillus species, rather they are involved in the stringent response and can in this respect govern the specificity of RNA polymerase (Nishino et al., 1979). Levels of ppGpp and pppGpp during outgrowth are the same as those found in vegetative cells (Setlow, 1974).

Cyclic nucleotides are also possible effectors in differentiation and although cyclic AMP (cAMP) is absent from the genus Bacillus (Setlow, 1973b) cyclic GMP (cGMP) is found in bacilli and the levels fluctuated as cells changed from log-phase to stationary phase growth (Bernlohr et al., 1974; Cook et al., 1980). The later feature makes c GMP an attractive candidate for a regulatory role in differentiation. Setlow and Setlow (1978) examined the levels of c GMP in dormant and outgrowing spores and vegetative cells and found that cGMP levels were very low during vegetative growth and cGMP was present at less than one molecule per spore during germination and outgrowth, therefore it seems

unlikely that cGMP is involved directly in outgrowth. Other nucleotides may be involved in the regulation of morphogenesis, for example GTP, Freese et al. (1978) have shown that B. subtilis can sporulate in the presence of excess glucose, ammonia and phosphate by a partial reduction in the synthesis of purine nucleotides. Furthermore, the levels of ATP and GTP can influence expression of genes both in vitro and in vivo and the rate of initiation of transcription is a linear function of ATP or GTP concentration (Nierlich, 1978). Similarly AMP can stimulate transcription by B. subtilis RNA polymerase (Badaracco et al., 1981). The levels of all these nucleotides change significantly during outgrowth (Section 2.1) and as well as influencing transcription directly will cause changes in the flux of metabolites through various metabolic pathways, so influencing the metabolic events occurring during outgrowth.

Therefore although there is as yet no small molecule that appears likely to be a candidate as a direct effector in the control of spore outgrowth, the levels of some nucleotides will influence the course of outgrowth.

4.4 Translational Control

While of lesser extent than transcriptional controls, regulation at the level of translation is known to play a part in bacteriophage development. To date there is no evidence to suggest that outgrowth is controlled to any extent at the level of translation, and the outgrowth mRNA population has a normal half life, although it is not possible to say if any individual messages have a longer or shorter half life than normal (Buu & Sonenshein, 1975; Kobayashi et al., 1965). Post-translational controls e.g. phosphorylation, adenylation, methylation or acylation have not been investigated in outgrowing spores and therefore no assessment of their part can be made, although this may be a profitable area for further study.

5. Outgrowth of Clostridium species

Little work has been done on the outgrowth of spores from anaerobic spore formers, probably because of the inherent difficulties involved in working with them. They are mentioned briefly to indicate that many of the features of outgrowth occurring in *Bacillus* species apply in the genus *Clostridium*. The spores of *Clostridium* are resistant to heat, radiation and a variety of chemical agents (Roberts & Hitchins, 1969) and contain dipicolinic acid (Murrell, 1969). During germination and outgrowth they behave in a very similar manner to *Bacillus* spores with regards to utilization of PGA, tRNA for nucleotides and degradation of spore-specific proteins (Hausenbauer *et al.*, 1977; Setlow & Waites, 1976). Macromolecular synthesis was also similar, with RNA and protein synthesis starting early after germination on swelling of the spore, but DNA synthesis commenced at the beginning of elongation, slightly earlier than in *Bacillus* species (Waites & Wyatt, 1974).

The outgrowth of other spore forming genera including among others *Sporosarcina*, *Thermoactinomyces* and *Desulfotomaculum* is beyond the scope of this introduction, especially since much work is needed on the many genera before any comparison can be made.

6. Aims and approach

The aim of this project was to analyse controls operating during spore outgrowth. The first step in such an analysis requires identification of an event specific to the process. The approach that was used here involved inhibitors which affect spore outgrowth preferentially (i.e. they do not affect other life-cycle stages to the same extent as outgrowth). These inhibitors provide probes to examine biochemical changes during differentiation. Once the nature of the preferential inhibition is established the inhibitor can be used as a marker for the biochemical change and more importantly a basis for a more detailed study of the biochemical event is provided.

Chapter 2

Materials and Methods

Organisms. Bacillus subtilis strains 168 (trp C2) and ts 134 (trp C2, thy, dna B) were supplied by Professor J. Mandelstam (University of Oxford). Bacillus subtilis strains W23 (6), B. subtilis var globigii (621), Bacillus cereus strain T (629) were supplied by Professor G.W. Gould (Unilever Research, Sharnbrook, Bedford). The number refers to the Colworth Bacterial Culture Collection (CBCC). Bacillus megaterium strains Texas and ATCC 9885 were supplied by Dr. I.W. Dawes (University of Edinburgh).

Media. Potato Glucose Yeast Extract (PGYE) sporulation medium of Dring and Gould (1971) contained in $g.l^{-1}$: potato extract, 4; glucose, 2.5; and yeast extract, 4. The pH was adjusted to 7.4, solid medium was prepared by the addition of $20gl^{-1}$ agar.

Minimal Salts Medium, a defined growth medium by Anagnostopoulous and Spizizen (1961) contained in $g.l^{-1}$: ammonium sulphate, 2; dipotassium hydrogen orthophosphate, 14; potassium dihydrogen orthophosphate, 6; trisodium citrate $2H_2O$, 1; and magnesium sulphate. $7H_2O$, 0.2. L-tryptophane ($0.02\ g.l^{-1}$) and glucose ($0.5g.l^{-1}$) were filter sterilized and added after the medium was autoclaved, pH was adjusted to 7.0.

Defined Outgrowth Medium, a defined outgrowth medium described by Kennett and Sueoka (1971) consisted of minimal salts medium and the following amino-acids in $mg.l^{-1}$: L-alanine, 50; L-arginine, 20; L-asparagine, 50; L-glutamate, 100; L-histidine, 50; L-leucine, 5; L-isoleucine, 10; L-methionine, 5; L-serine, 5; L-threonine, 20; and L-valine, 20. The amino-acids were filter sterilized and added after the medium was autoclaved.

Low Phosphate Medium, a low phosphate medium described by Tempest et al. (1968) contained in mM: ammonium sulphate, 50; sodium dihydrogen

phosphate, 1.5; di potassium sulphate, 3; and citric acid, 1.L-tryptophane ($0.02g.l^{-1}$) and glucose ($0.5g.l^{-1}$) were filter sterilized and added after the medium was autoclaved, pH was adjusted to 7.
Nutrient Broth (NB), Nutrient Broth no. 2 (Oxoid) was used routinely, solid medium was prepared by adding 1.5% (w/v) agar.

Spore preparation. Spores were prepared in large trays by the method of Dring and Gould (1971) on solidfied PGYE. After harvesting, vegetative cells were removed using lysozyme (200 ug ml^{-1} in 0.85% (w/v) NaCl) and the preparation washed at least eight times with cold distilled water. The spores were stored in distilled water at $4^{o}C$.

Cultivation. Unless otherwise stated, all incubations were done at $30^{o}C$ with shaking. Before germination and outgrowth experiments, spores were heated at $70^{o}C$ for 20 min to kill any germinated spores. The heated spores (approximately 10^{8}) were suspended in 10ml of medium in 100ml flasks. Septation was visualized by adding CsCl to a drop of culture before microscopic examination. The course of germination, outgrowth and vegetative growth was monitored by measuring turbidity changes at 600nm.

Sporulation. Sporulation was studied using the resuspension method of Sterlini and Mandelstam (1969). Bacteria were grown to a turbidity of about 0.5 (0.2mg dry wt. ml^{-1}) in the casamino-acids medium of Donnellan et al. (1964) supplemented with 20ug ml^{-1} tryptophan and no glucose. The cells were harvested by centrifugation, resuspended in pre-warmed sporulation medium, without glucose (Donnellan et al., 1964) and incubated

at 37°C with vigorous shaking. After fixation with formaldehyde, sporulation was estimated by counting phase-bright spores using phase-contrast microscopy. One hundred spores or 1000 vegetative cells were counted, (whichever was least) and values for sporulation were expressed as the percentage spores in the total population of both spores and vegetative cells counted.

Glucose - 6 - phosphate dehydrogenase assay. Glucose - 6 - phosphate dehydrogenase (GPDH) (E.C.1.1.1.49) was assayed at 25°C in a 3ml reaction mixture consisting of: tri-ethanolamine buffer, pH 7.2, 8.3mM; glucose - 6 - phosphate, 1.2mM; and nicotinamide adenine dinucleotide phosphate (NADP) 0.37mM. The reaction was started by adding lug of enzyme preparation to the cuvette, absorbance was measured at 366nm, over five minutes. $MgCl_2.7H_2O$ and chloroquine disphosphate were added at the concentrations shown in the results. Initial velocities were estimated from tangents to the recorded curve.

Isotope incorporation. The incorporation of precursors into protein, RNA and DNA was measured by resuspending spores in defined outgrowth medium containing: [u - ^{14}C] protein hydrolysate (Amersham CFB.25), 0.5u $Ci.ml^{-1}$; [5, 6 - ^{3}H] uracil, 1u $Ci.ml^{-1}$ (1ug uracil ml^{-1}); and [6 - ^{3}H] thymidine, 1uCi.ml^{-1} (5ug thymidine ml^{-1}) with 250ugml^{-1} 2-deoxyadenosine to improve the fidelity of thymidine labelling of DNA (Rana & Halvorson, 1972a). Culture samples were added to cold trichloro-acetic acid (TCA, 10% w/v final concentration) containing excess non-radioactive carrier. The resulting precipitates were collected by filtration on glass fibre filters (Whatman GF/A). These were washed with 25ml of cold 5% (w/v) TCA containing excess carrier, dried and inserted into vials containing 5ml of scintillant (0.5% w/v) 2.5 diphenyloxazole and

0.1% (w/v) dimethyl - 1, 4-bis-2-(5-phenyloxazolyl) benzene in toluene). ^{3}H and ^{14}C counts were determined in a Beckman liquid scintillation counter. Uptake of [5 - 6 - ^{3}H] - Uracil was determined by collecting cells on 0.45u Millipore filters and rapidly washing with warmed outgrowth medium containing excess cold carrier, dried filters were counted as above.

DNA-dependent RNA polymerase assay in permeabilized cells. Vegetative cells ($10^{8}ml^{-1}$) and outgrowing spores ($10^{8}ml^{-1}$) were harvested from 10ml NB by centrifugation, rapidly resuspended in an equal volume of ice cold 0.05M Tris-hydrochloride, pH8; recentrifuged at $4^{o}C$ and finally resuspended in 1ml of the same buffer. After holding the suspension on ice for 10 min, DNA-dependent RNA polymerase activity was assayed by measuring the incorporation of [5, 6 -^{3}H] - uridine triphosphate (UTP, $2uCi.ml^{-1}$) into TCA-insoluble material according to the method of Fisher et al. (1975).

Isolation of cell membranes. Vegetative cell membranes were prepared from mid-logarithmic phase cells harvested by centrifugation and treated to form vesicles; 2mM phenylmethysulfonyl flouride (PMSF) was present throughout the preparation to inhibit serine protease activity (Konings et al., 1973). Membranes from dormant and outgrowing spores were isolated by the method of Weber and Broadbent (1975), including the lysozyme and RNase treatment used by Konings et al. (1973). Membranes were washed by centrifugation three times with 0.1M potassium phosphate buffer (pH.6) containing 10mM EDTA

SDS-polyacrylamide gel-electrophoresis of membrane proteins. Discontinuous gel-electrophoresis was done according to the method of Laemmli (1970) with an acrylamide gradient of 7% to 20% (w/V). Na N lauroyl

sarcosinate (1% w/v) was included in the sample buffer to solubilize the membranes. Gels containing 200ug of protein were stained for 2h with Kenacid blue R (0.0025% (w/v) in methanol: glacial acetic acid: water, in the v/v ratio of 45:10:45 and destained with several changes of methanol: glacial acetic acid: water, 5:7.5:87.5 (v/v) at 30°C.

Chloroquine uptake. The uptake of [Ring - 3 - ^{14}C] chloroquine diphosphate was measured following the method of Kashket and Barker (1977). Spores (2.8×10^8 ml^{-1}) were grown out in NB at 30°C and 0.1uCi.ml^{-1} [^{14}C] - chloroquine added, cells were separated from the medium by layering a 0.25ml sample over 0.5ml of a mixture of silicone oils (25% v/v Fluid 510, 50 centistokes and 75% v/v Fluid 550, Dow Corning) in a 1.5ml Eppendorf centrifuge tube and centrifuging for 1 min in an Eppendorf Microfuge. The upper aqueous supernatant layer and part of the oil layer were removed with a Pasteur pipette. The bottom of the tube containing the cell pellet was cut off with a razor blade and placed in 5ml Triton toluene scintillant before counting. Vegetative cells were treated in the same way; viable cell counts were estimated for each experiment.

Protein estimation. Protein assays followed the method of Lowry et al. (1951) using bovine serum albumin standards.

DNA estimation. DNA was assayed by the method of Burton (1956) using 2-deoxyadenosine standards.

RNA estimation. RNA was estimated following the procedure of Herbert et al. (1971) using ribose standards.

Addendum

Estimation of minimum inhibitory concentrations (mic). For germination and outgrowth heat treated spores (10^8/l) were added to 10ml of medium containing various concentrations of inhibitor and incubated at 30°C with shaking. Subsequent germination and outgrowth of spores was followed by removing 0.25 ml aliquotes of the culture and turbidity changes were measured at 600nm. The mic for germinating spores was considered to be the lowest concentration of inhibitor which prevented a decrease in the initial turbidity of the culture. The lowest concentration of which prevented post-germinative swelling and subsequent emergence of the germ-cell from the spore coat was the mic for outgrowth. For vegetative cultures, 1 ml of a logarithmic phase culture was inoculated into 9 ml of pre-warmed medium containing various concentrations of inhibitor and growth was followed as above. The mic was that concentration of inhibitor which prevented a further increase in turbidity after a period of incubation. The alkyl-p-hydroxybenzoates were only soluble in aqueous solutions at low concentrations, therefore, to obtain high concentrations stock solutions, they were dissolved in 50% ethanol and diluted at least 50-fold in the assay medium. Since ethanol itself inhibited germination, outgrowth and vegetative of B. subtilis, it was necessary to obtain an extrapolated value for the mic of the alkyl-p-hydroxybenzoates from dilutions of inhibitor where the inhibitory effect of ethanol was absent. The percentage inhibition was calculated as follows: Inhibition (%) = (I/C) x 100; where I is the percentage change in turbidity of the system with inhibitor and C that of the control system. The mic was estimated from a standard curve of the values obtained in the presence of inhibitor (Watanabe & Takesue, 1976).

In all cases the mic values presented were the average of three separate determinations and in no case did individual values vary by more than 5%.

Mutagenesis and isolation of chloroquine resistant mutants. Mutations were induced with N-methyl-N' nitro-N-nitrosoguanidine (NTG), ethylmethanesulphate (EMS) and ultraviolet light (UV). For mutagenesis by NTG vegetative cells of B. subtilis were grown in NB containing 50 ug ml^{-1} NTG for 1h at $30^{o}C$. Vegetative cells grown in NB were collected by filtration (Millipore 0.45 u pore) and resuspended in sterile 0.01M tris (hydroxymethyl)-amino-methane/0.01M maleate buffer, pH6.0, before treatment with EMS. The cells were incubated with 0.3% (V/V) EMS for 1h at $30^{o}C$ to give approximately 30% kill. After both treatments the cells were washed three times with NB and grown in NB at $30^{o}C$ for several generations to allow recovery. Mutagenesis by U.V. light was done with vegetative cells grown in minimal medium. A sample of culture in a petri-dish was exposed to 12,000 erg/mm^2 from a germicidal lamp (90% kill). Aliquots of culture were inoculated into NB and incubated for several generations in the dark. In all cases spore stocks were prepared from these mutagenized cultures as described above. To isolate mutants resistant to chloroquine during spore outgrowth, heat treated (10^8) were spread on solidified NB containing 500 ug ml^{-1} chloroquine and incubated at $30^{o}C$ to allow colonies to develop. Alternatively, 10^8 spores were resuspended in 10ml NB containing 500 ug ml^{-1} chloroquine, incubated overnight at $30^{o}C$ samples were plated onto solidified NB containing chloroquine (500ug ml^{-1}) and incubated at $30^{o}C$. In neither case were any mutants, resistant to chloroquine, isolated. Vegetative cells derived from the mutagenized spore preparations were inoculated into NB containing 5mg ml^{-1} chloroquine, incubated overnight and an aliquot reinoculated into NB supplemented with chloroquine (5mg ml^{-1}) for further incubation. Samples were removed at intervals and plated onto solid NB. No mutants resistant to chloroquine (5mg ml^{-1}) during vegetative growth were isolated by this method. However, some spontaneous mutants resistant to chloroquine during vegetative growth were isolated after several cycles of grov

in the presence of 5mg ml^{-1} chloroquine, including CQR1 (see Chapter 8). The mutants obtained were stored at $4^{o}C$ on solid NB.

Chemicals. Radiochemicals, [U - ^{14}C] protein hydrolysate (59 m Ci. milliatom^{-1}), [5, 6 - ^{3}H] uracil (55 Ci. m mol^{-1}) [5 -6^{3}H] uridine triphosphate (40 Ci. m mol^{-1}) and [6 - ^{3}H] thymidine (2 Ci. m mol^{-1}) were obtained from Amersham; [Ring - 3 - ^{14}C] chloroquine diphosphate (30 m Ci. m mol^{-1}) was from New England Nuclear. Acriflavin, 2, 4 - dinitrophenol, Kenacid blue R, methylamine phenethylalcohol and rotenone were from B.D.H. Ltd. N, N-dicyclohexyl carbodiimide was from Cambrian chemicals, Beddlington Farm Road, Croydon. Nisin was from Koch-Light. Tetrachlorosalicylanilide was obtained from Eastman Kodak. Acrylamide, 5-chloro-8-hydroxyquinoline, m-chlorophenylhydrazone, chloroquine diphosphate, erythromycin, ethidium bromide, nalidixic acid, novobiocin, polymyxin, quinacrine, rifampicin, tylosin tartrate and valinomycin were purchased from Sigma. Parahydroxy benzoate esters were a gift from G.J. Dring (Unilever Research). Other reagents were analytical reagents grade where possible.

Chapter 3

The Search for Preferential Inhibitors of Spore Outgrowth

Introduction

Spore outgrowth is a process of differentiation in which a biosynthetically dormant structure initiates synthesis and develops a fully vegetative complement of functions in an ordered manner. The controls operating during outgrowth are of great interest but before these can be understood it is necessary to identify and order events which are specific to or differ during outgrowth.

Several approaches can be used to analyse these events. Conditional mutations affecting outgrowth can be studied to determine the nature of sequential control during outgrowth and the inter-relationship between the processes of outgrowth and vegetative growth (see Section 3.3). Several inhibitors exist which affect spore outgrowth preferentially (Strange & Hunter, 1969; Gottfried *et al.*, 1979). These provide tools to examine biochemical changes during outgrowth, and can be used to select for relevant mutations.

A number of inhibitors have been screened to see if they preferentially inhibit outgrowth, that is to find any inhibitor which shows a large difference in its effect on spore outgrowth compared with vegetative growth and which acts on spores of a range of *Bacillus* species.

Screening of Inhibitors

In the basic experiment, for each inhibitor the minimum inhibitory concentration (mic) that prevented the development of outgrowing spores was determined and vegetative cells were tested with the inhibitor at its outgrowth mic. Table 6 shows the mic of various inhibitors for both spore outgrowth and vegetative growth. Several of the inhibitors showed a preferential action on outgrowth, i.e. acriflavin, chloroquine, novobiocin and rifampicin. All gave small differences in mic for outgrowth and vegetative growth except for chloroquine for which the difference was tenfold; spores being less resistant than vegetative cells, and thus chloroquine was of particular interest because of its large differential effect.

Effect of nisin and tylosin

The inhibition shown by nisin and tylosin were interesting. Gould (1964) has shown that nisin prevented outgrowth at a concentration of 10 units ml^{-1} but vegetative cells were often resistant to 100 units ml^{-1}. In this study it was found that although there was a preferential inhibition of outgrowth by nisin at low cell density, by increasing the concentration of vegetative cells no preferential inhibition was evident (Fig. 7). The reason for this is unknown but may be due to either titration or deactivation of the antibiotic, the later explanation is more likely since increasing the spore density did not appear to change the mic of outgrowth.

Tylosin, an ester of nisin, prevented first septation of the outgrowing spore but not outgrowth *per se* at lower concentrations (2ug ml^{-1}); this concentration only inhibited vegetative growth by about 50% in

150 min. Increased concentrations of tylosin (10ug ml^{-1}) prevented post-germinative swelling of the spore and vegetative growth in agreement with Gould (1964). Therefore these inhibitors, while they may prove to be useful probes for outgrowth may involve factors which complicate their use.

Chloroquine inhibition

McDonald (1967) first reported the preferential inhibition by chloroquine of spore outgrowth in B. subtilis; he found 500ug ml^{-1} prevented outgrowth whereas 5mg ml^{-1} was required to inhibit vegetative growth. These results were confirmed here, chloroquine at a concentration of 500ug ml^{-1} completely inhibited outgrowth of B. subtilis spores (Fig. 8) but had little effect on spore germination (Fig. 9) or on vegetative cell growth as measured by turbidity increase (Fig. 10). A tenfold increase in chloroquine concentration (5mg ml^{-1}) inhibited vegetative growth completely after 90 min and thereafter the cells lysed (Fig. 10).

Table 6

Mic of various inhibitors on spore outgrowth and vegetative growth in Bacillus subtilis

Inhibitor		Mic (ug ml^{-1}) Outgrowth	Vegetative Growth	Mode of Action	References
Acriflavin		5	5 (≈90%)	Intercalates with DNA	Franklin & Snow (1975)
Chloroquine	1	500	5000	Intercalates with DNA	McDonald (1967)
Erythromycin		0.5	0.5	Binds to ribosome	Franklin & Snow (1975)
Ethidium bromide		5	5	Intercalates with DNA	Franklin & Snow (1975)
5-chloro-8-hydroxy-quinoline		5	5	Chelates cations	Frier (1971)
Nisin	1	8 (units)	8 (units)	Unknown	Gould (1964)
Novobiocin	1,2	6	7	Inhibits DNA gyrase	Gottfried et al.(1979)
Para-hydroxy-benzoate (esters)	2			Membrane active	Watanabe & Takesue (1976)
Methyl		11.5mM	11.5mM		
Ethyl		4.0mM	4.0mM		
Propyl		2.5mM	2.5mM		
Butyl		0.8mM	0.8mM		
Quinacrine		50	50	Intercalates with DNA	Franklin & Snow (1975)
Rifampicin		1.5	1.5 (≈85%)	Inhibits RNA-polymerse	Franklin & Snow (1975)
Tylosin	1	10	10	Unknown	Gould (1964)

1 Reported as preferential outgrowth inhibitors. 2 More detailed study in chapter 6.

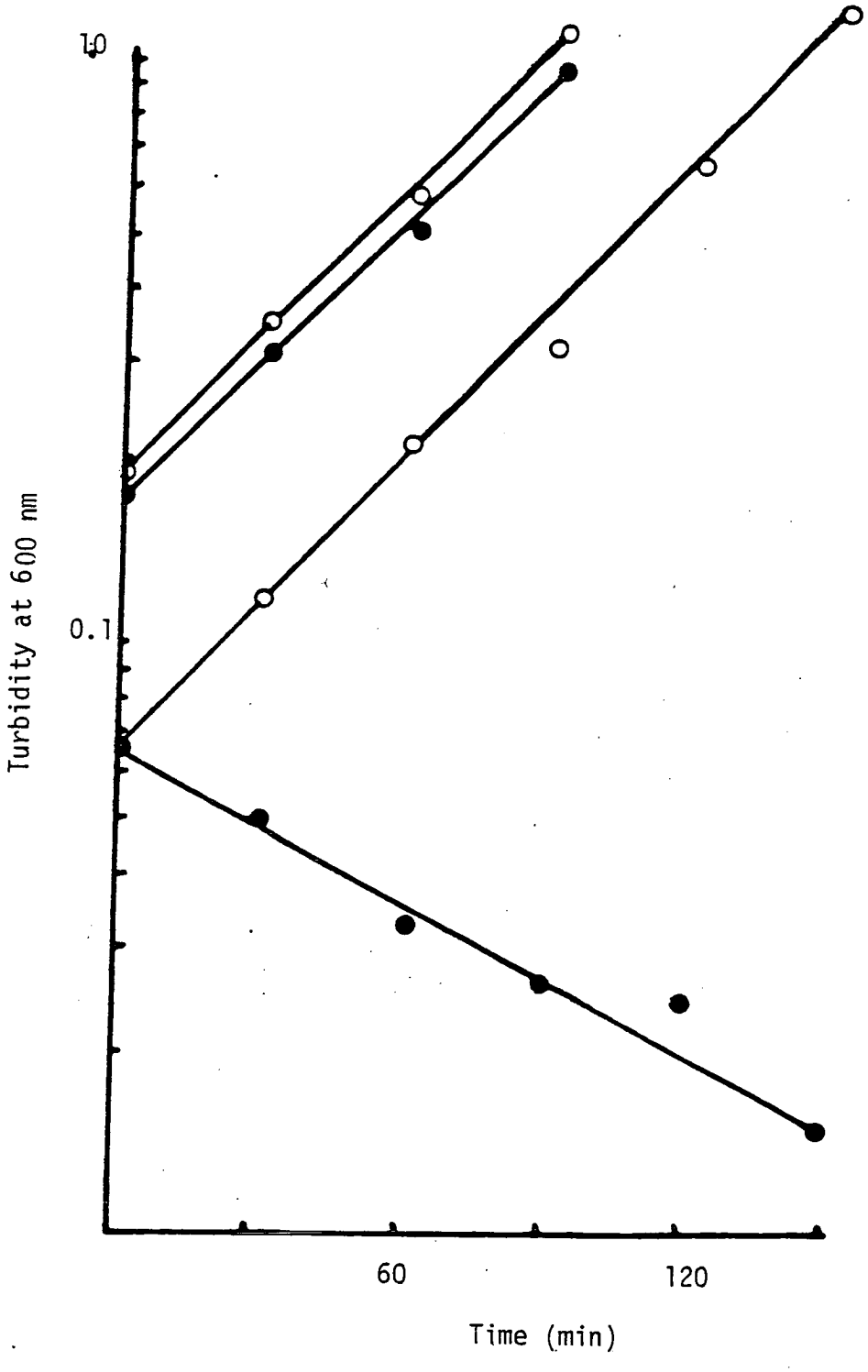


Figure 7. Effect of cell density on nisin inhibition of vegetative growth of B. subtilis. Cells were grown in NB at 37°C, nisin was added at zero time: (○), no nisin; (●), 10 units ml^{-1}.

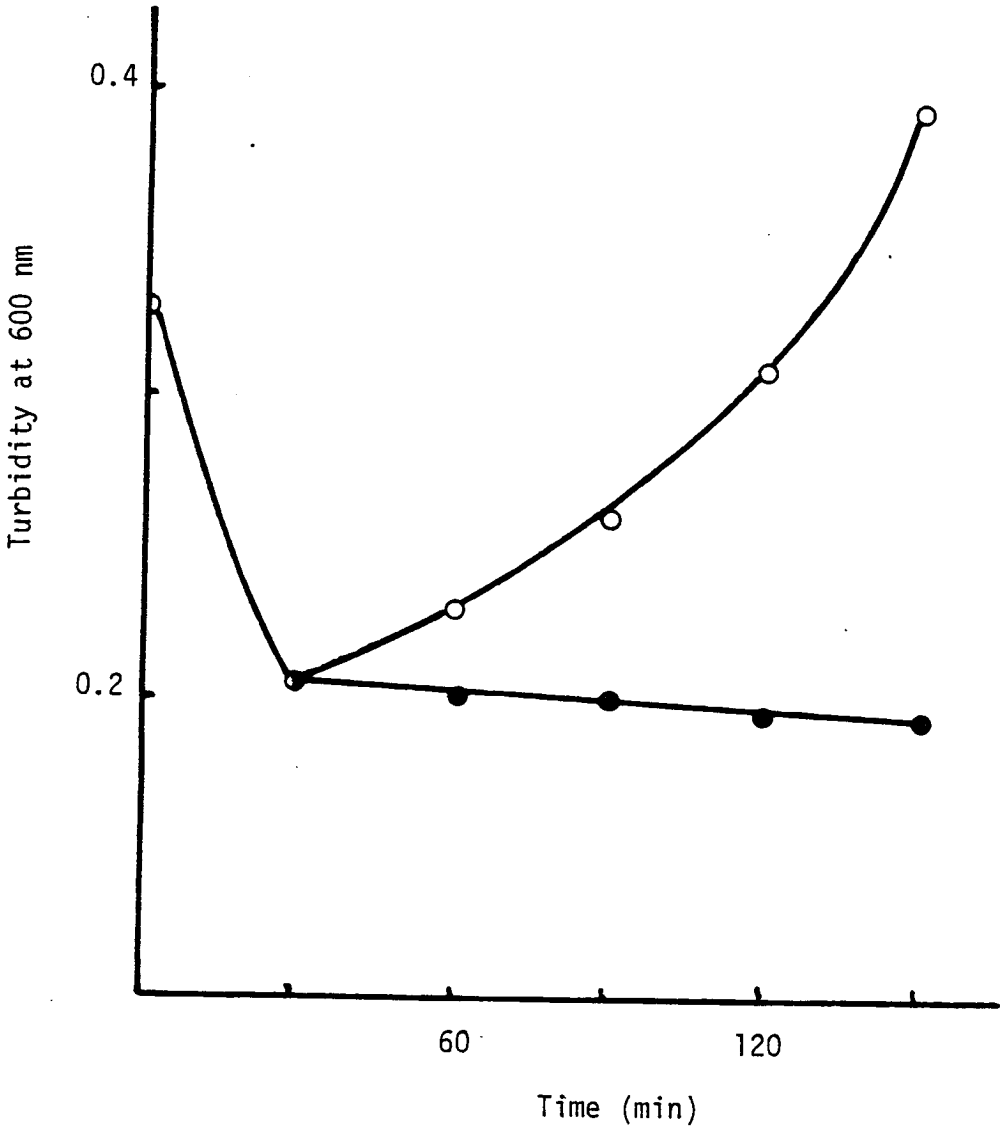


Figure 8. Effect of chloroquine on spore outgrowth. 10^8 spores ml^{-1} were added to 10ml NB and incubated with shaking at 30°C: (○), no chloroquine; (●), 500ug ml^{-1} chloroquine added at zero time.

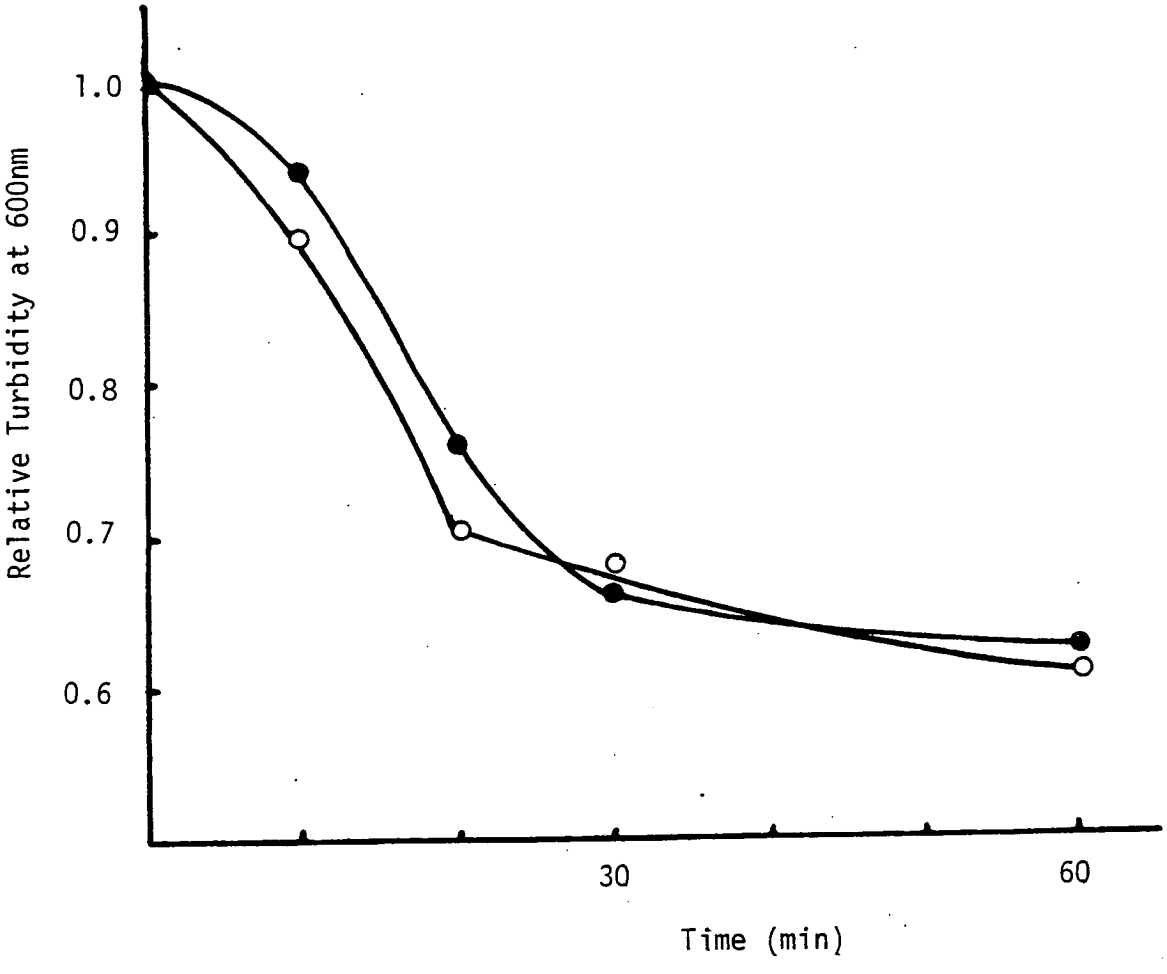


Figure 9. Effect of chloroquine on spore germination. 10^8 spores were added to 10ml of 100mM Na phosphate buffer, pH7 containing 10mM L-alanine and were incubated at 30°C: (○), no chloroquine; (●), 500ug ml^{-1} chloroquine added at zero time.

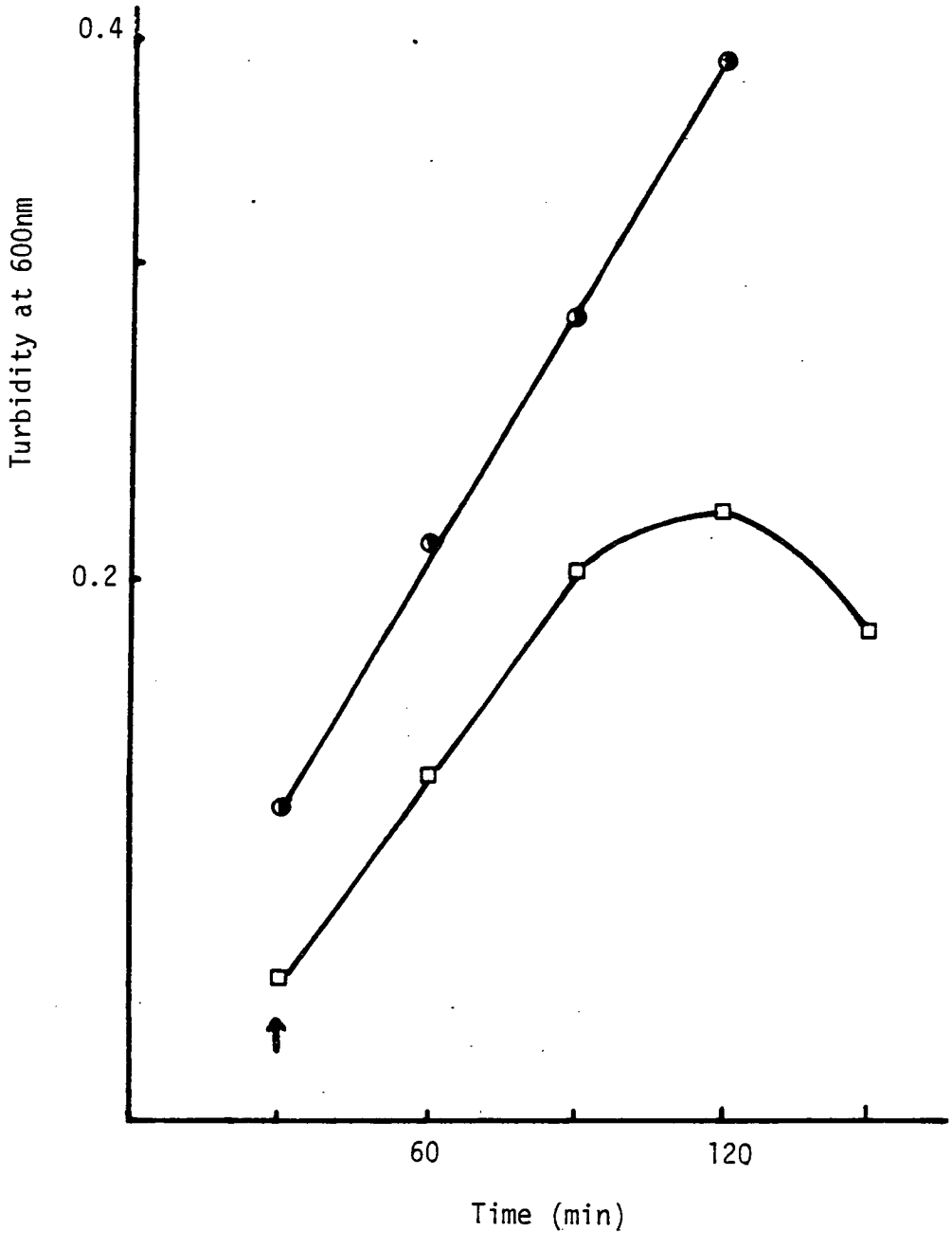


Figure 10. Effect of chloroquine on vegetative growth. Legend as in Fig. 7, incubation at 30°C: (◑), no chloroquine and 500ug ml^{-1} chloroquine; (□), 5mg ml^{-1} chloroquine. Chloroquine was added at the time indicated by the arrow.

Increasing the concentration of spores or vegetative cells two-to threefold did not alter the pattern of chloroquine inhibition. Sporulation was completely inhibited by 500ug.ml^{-1} of chloroquine (data not shown).

The preferential inhibition of outgrowth did not appear to be species specific. Table 7 shows the mic of chloroquine for outgrowth of a number of Bacillus species representing spores of both large-celled species which open by lysis of the spore coat and small-celled species which open by rupture of the coat (Lamanna, 1940).

In both cases outgrowth was inhibited preferentially by chloroquine, although B. megaterium Texas had an increased mic for outgrowth.

Table 7

Effect of chloroquine on spore outgrowth of various Bacillus species

Organism	Minimum inhibitory concentration	
	Outgrowth (mg ml^{-1})	Vegetative growth (mg ml^{-1})
*B. cereusT**	0.5	>5
*B. megaterium** ATCC 9885	0.5	>5
*B. megaterium** Texas	2.0	>7
B. subtilis 168	0.5	5
B. subtilis 621	0.5	3
B. subtilis W23	0.5	4

* The first three organisms are large celled species.

Determination of mic was described earlier. Concentrations of chloroquine much above 5mg ml^{-1} caused the medium to precipitate and cells to flocculate.

Discussion

Several inhibitors examined here have been found to inhibit spore outgrowth preferentially. In the case of inhibitors for which the differences in mic between outgrowth spores and vegetative cells is small, e.g. acriflavin, novobiocin and rifampicin, the significance of the preferential inhibition is doubtful. However, novobiocin has been reported as a preferential inhibitor of spore outgrowth (Gottfried et al., 1979).

Nisin and tylosin also showed a preferential inhibition of outgrowth. In the case of nisin this was found to depend, in part, on the density of the cell population during vegetative growth and may be due to inactivation of the antibiotic. Gould (1964) has shown that up to 20 fold greater concentrations were required to inhibit vegetative growth than outgrowth, although the method used to determine the mic would not have shown the effect of increasing cell concentration in increasing the mic.

Tylosin, at lower concentrations specifically prevented septation of the outgrowing spore but not swelling, emergence and elongation of the germinated spore, confirming the work of Gould (1964). The specific inhibition of first septation at lower concentrations of tylosin is particularly interesting, septation of the outgrowing spore is, by definition, not part of outgrowth (Strange & Hunter, 1969) but in the absence of any preferential uptake of tylosin by spores, first septation differs from later cell divisions and therefore is not identical with the vegetative process. Tylosin may prove a useful inhibitor in studies of cell septation.

Outgrowing spores showed a tenfold increased sensitivity to chloroquine compared to vegetative cells. This large difference in mic was independent of both cell concentration and species of Bacillus tested and this makes chloroquine an attractive inhibitor for studies of spore outgrowth. The basis of the preferential inhibition may be due to differences in its uptake, its target site or inactivation of the drug by vegetative cells and these possibilities will be considered in later chapters.

It is also important to establish, as far as possible, which function(s) are affected by chloroquine in both vegetative cells and outgrowing spores, and hence the basis of inhibition by chloroquine is examined in the following two chapters.

Chapter 4

The Timing and Basis of Chloroquine Inhibition

Introduction

For chloroquine to be useful as a biochemical probe of functions that change during outgrowth it is necessary to identify its mode of action. Numerous cellular functions have been reported to be affected by chloroquine (Fig. 11); most of the studies of the drug have been concerned with its antimalarial properties.

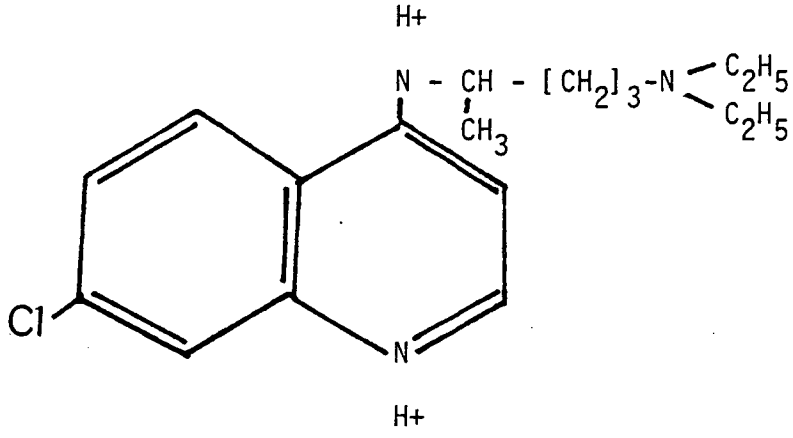


FIGURE 11. The structure of chloroquine.

In Plasmodium knowlesi chloroquine (100nM) was found to inhibit both DNA and RNA synthesis and to a lesser extent protein synthesis (Polet & Barr, 1968; Gutteridge et al., 1972), and the basis of this inhibition probably lies in the ability of chloroquine to intercalate with DNA (Ciak & Hahn, 1966; Waring, 1970; Gutteridge et al., 1972). Also it has been shown that chloroquine treated P. knowlesi, in a monkey host, degrades ribosomal RNA after the development of a drug-induced autophagic vacuole in the cell, possibly due to an activation of parasite nucleases (Warhurst & Williamson, 1970). Also chloroquine (1.5mM) can induce a conformational change in ribosomes which may affect protein synthesis

(Hultin, 1970).

Several studies have been made on the effect of chloroquine on the *in vitro* synthesis of DNA, RNA and protein. Chloroquine (5mM) inhibited DNA polymerase activity completely while RNA polymerase was inhibited to a maximum extent of 50% by various concentrations of the drug and the inhibition of both enzymes was dependent on the amount of DNA template present and independent of enzyme concentration (Cohen & Yielding, 1965a; O'Brien *et al.*, 1966). These studies provided evidence that the inhibitory effect of chloroquine on DNA and RNA synthesis was due to the drug binding to the DNA template but Whichard *et al.* (1972) found chloroquine inhibition of polymerase activity was not independent of enzyme concentration and it is possible chloroquine binds to the DNA and RNA polymerase molecules. Protein synthesis in a rat liver cell-free system was inhibited by chloroquine (0.5mM) only if the drug was incubated with the artificial template poly-Uracil (poly-U) before adding it to the system; the drug had no effect if it was added after the poly-U. It is considered unlikely that chloroquine inhibits protein synthesis *in vivo*, the observed inhibition is probably due to the effect of the drug on RNA synthesis (Roskoski & Jaskunas, 1972). The concentrations of chloroquine used in *in vitro* studies probably fall into the range effective against *P. knowlesi* since the parasite can concentrate the drug 1000 fold from its environment (usually 100nM) (Fitch, 1969).

Although there is strong evidence that the mode of action of chloroquine is related to its ability to intercalate into DNA, several other inhibitory modes for chloroquine have been reported. Inglot and Wolna (1968) have shown that chloroquine (5mM) caused lysis of erythrocytes.

Chloroquine can also inhibit the activity of thiol-containing enzymes e.g. alcohol dehydrogenase, at a concentration of 0.35mM; the inhibitory effect could be reversed by thiol reagents (Fiddick & Heath, 1967). This property of chloroquine may explain the observed dependence of drug inhibition of DNA and RNA polymerase activity on enzyme concentration as discussed above. Glucose-6-phosphate dehydrogenase activity was inhibited by chloroquine at low $NADP^+$ concentrations but not at higher concentrations of $NADP^+$ (Cotton & Sutorius, 1971). The latter observation may be the cause of the decreased flux of glucose through the hexose monophosphate shunt observed by Kelmen et al. (1981) in erythrocytes in the presence of 1mM chloroquine.

The above studies were concerned with the action of chloroquine on eukaryotic organisms; does chloroquine have the same effect in prokaryotes? Bacillus megaterium was killed by chloroquine at a concentration of 1.5mM, the bacterium ceased to synthesize DNA and RNA after 20 minutes in the presence of the drug, rRNA was degraded, but respiration was unaffected by sub-lethal concentrations (Ciak & Hahn, 1966). The authors concluded the lethal effect was due to inhibition of DNA synthesis. Indeed it seems likely that the inhibitory effect of chloroquine on bacteria is due to the intercalation of chloroquine into DNA since the drug (3mM) can preferentially inhibit replication of a DNA-containing virus in Eschericia coli, and inhibit repair of UV-induced damage in the same organism (Yielding, 1967; Yielding et al, 1970). Similarly 0.5mM chloroquine caused reversion of frame-shift mutations in Salmonella typhimurium (Schupbach, 1979).

Overall, the main target of chloroquine in both eukaryotic and prokaryotic organisms appears to be DNA, although secondary effects on

membrane stability and various enzymes may be involved in the killing of cells. In this chapter, the phenomenon of preferential inhibition of outgrowth has been characterized more fully and an attempt made to establish the basis of chloroquine inhibition and development of resistance during spore outgrowth.

Timing of chloroquine inhibition

The first step in analysing outgrowth using specific inhibitors was to identify the point at which further development was prevented. Microscopic examination of outgrowing spore populations in the presence of chloroquine showed that normal post-germinative swelling occurred up to the point of germ-cell emergence from the spore but continued no further. This observation was confirmed turbidometrically on an outgrowing population of spores which had been pre-germinated in 0.1 M sodium phosphate buffer (pH 7.4) containing 10mM L-alanine, before resuspension in NB (Fig. 12). Normal swelling was seen up to 120 min after incubation. The pre-germination technique reduced the masking of turbidity changes caused by asynchronous germination, although outgrowth to septation (180 min) took longer than in a non pre-germinated culture.

The point at which the spore ceases to continue outgrowth in the presence of an inhibitor need not coincide with that at which the expression of the function is inhibited, similarly this function may only be required briefly for further development. The question of timing of the function that is inhibited can be answered in part by adding the inhibitor at various times during outgrowth. Figure 13 illustrates an experiment of this kind in rich medium. Within the

first 50 min of germination and outgrowth, the addition of 500ug ml^{-1} chloroquine prevented further outgrowth; after 50 min the spores became less sensitive to chloroquine and normal outgrowth was seen if chloroquine was added at 100 min, concurrent with germ-cell emergence. The degree of synchrony estimated by the presence of septate cells was about 90-95% at 150 min. In defined outgrowth medium, sensitivity to the drug was gradually lost from 120 min; full resistance developed around 150 mins and the outgrowth synchrony was 85-90% after 300 min (data not shown). Outgrowth of fresh spores resuspended in medium in which outgrowth had occurred was prevented by chloroquine, so the drug was not inactivated by the spores or by diffusible products produced during outgrowth (data not shown). From these results it appears that as outgrowth proceeds a function which is either: (i) sensitive to chloroquine,or (ii) prevents entry of the drug into outgrowing spores or (iii) detoxifies the drug,changes early during the process and so resistance develops.

Reversal of chloroquine inhibition

Chloroquine is not sporicidal because spores blocked during outgrowth with chloroquine, harvested and resuspended in fresh medium can continue outgrowth (McDonald, 1967). Vegetative cells eventually lyse in the presence of 5mg ml^{-1} chloroquine (see chapter 3) and lose gram-stainability after about 90 min cells are also unable to form colonies if replated from broth culture after about 180 min.

Can chloroquine inhibition be reversed? Media composition appeared to have little effect on the pattern of chloroquine inhibition

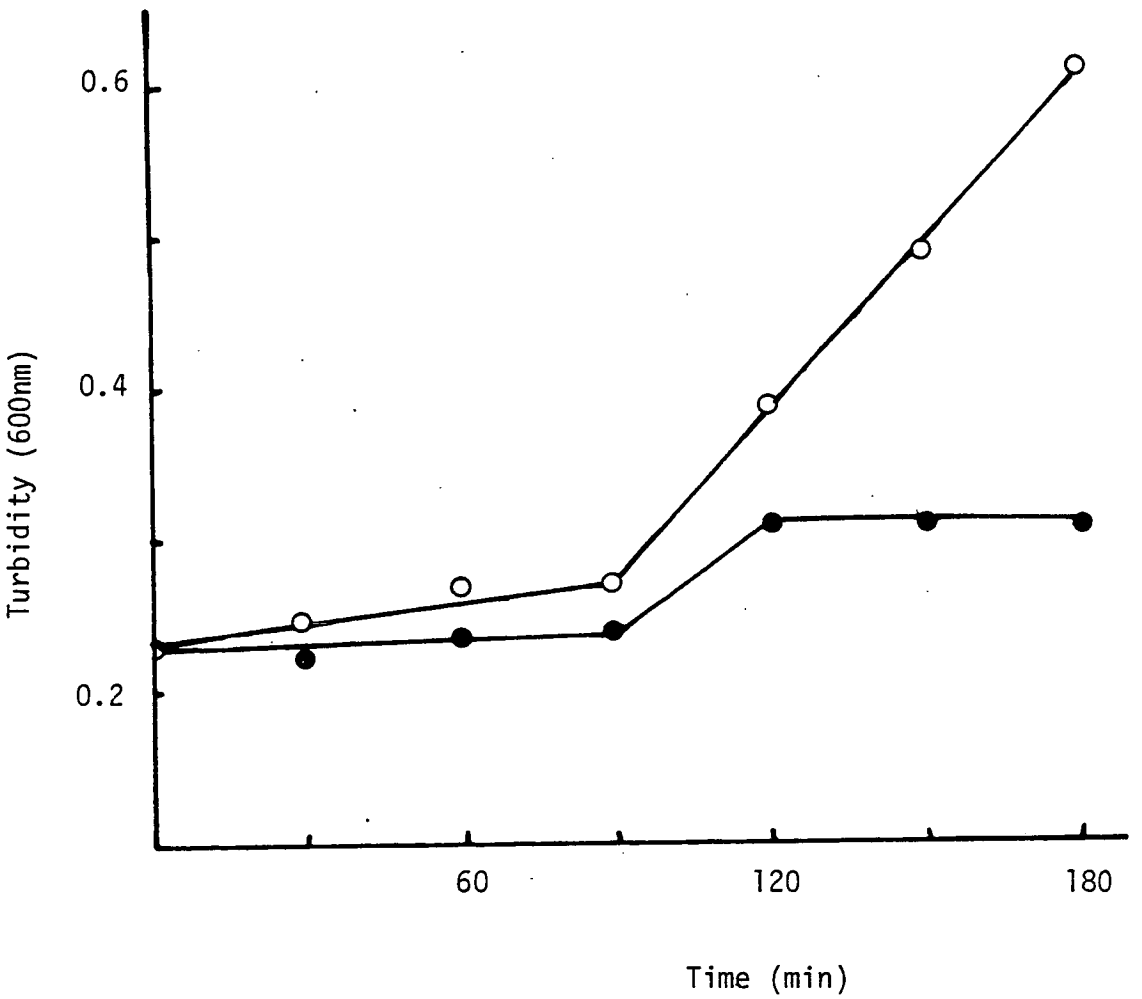


Figure 12. Effect of chloroquine on outgrowth of pre-germinated spores. 10^8 spores were pre-germinated at 30^oC for 1h in 0.1 M Na phosphate buffer (pH7.4) containing 10mM L-alanine, centrifuged and resuspended in NB: (○), no chloroquine; (●), 500ug ml^{-1} chloroquine added at zero time.

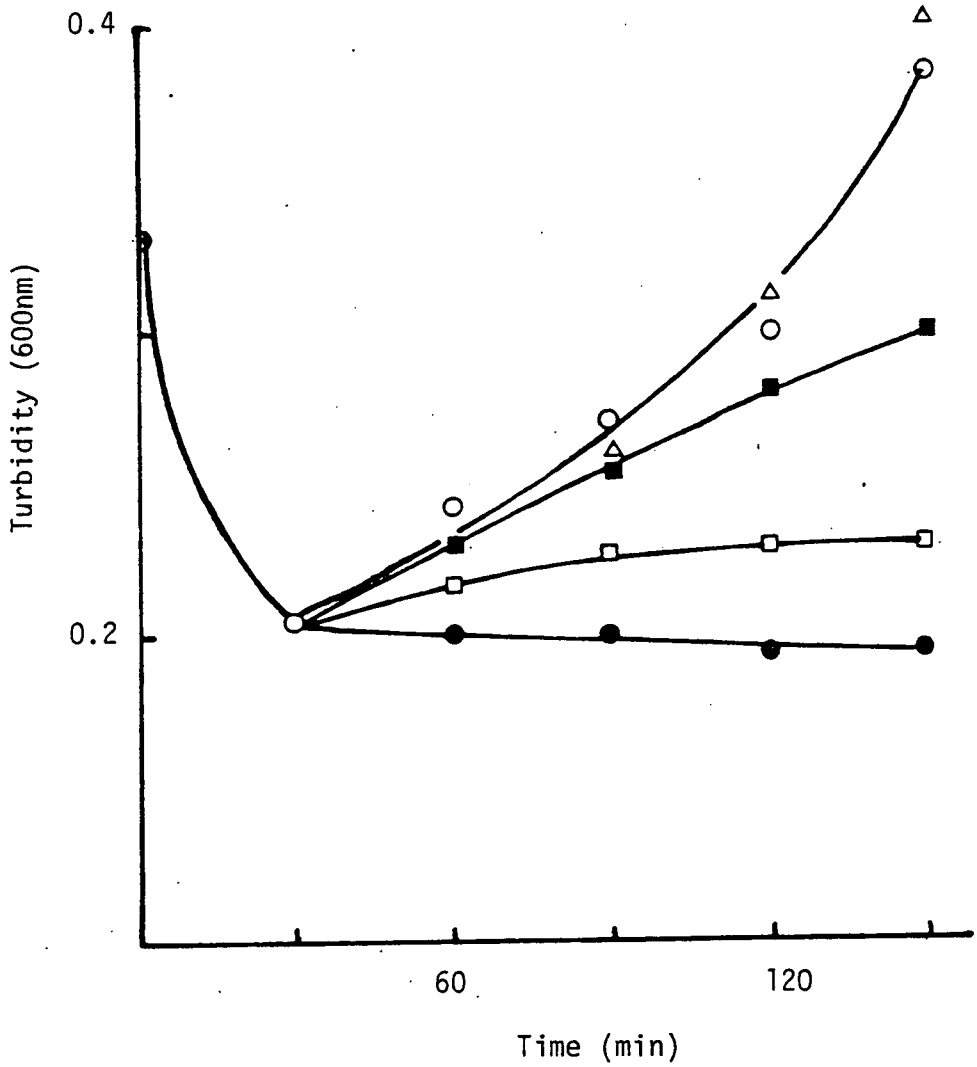


Figure 13. Timing of the development of chloroquine resistance during outgrowth. Spore outgrowth was measured as described previously. Chloroquine was added at: (●), omin; (□), 30 min; (■), 50 min; (○), 100 min; (△), no chloroquine.

of outgrowth or vegetative growth, and the drug inhibited spore outgrowth and vegetative growth in NB, penassay broth (Difco) or a defined minimal medium, therefore chloroquine is not mimicking or preventing synthesis of the more common amino-acids, purines, pyrimidines, vitamins or fatty acids.

Addition of 10-20% (W/V) sucrose protects osmotically fragile cells, and spores with coats stripped by the alkaline - SDS - dithiothreitol treatment of Aronson and Fitz-James (1968) show improved outgrowth in thepresence of 20% sucrose (Dawes, personal communication). Sucrose at 10 or 20% (W/V) does not relieve the inhibition of outgrowth or vegetative growth by chloroquine, therefore it is unlikely that chloroquine is acting as a surface active agent promoting osmotic fragility (Inglot & Wolna, 1968).

Chloroquine inhibition of thiol enzymes is reversible with thiol reagents (Fiddick & Heath, 1967), additions 0.5mM to 3mM dithiothreitol or cysteine did not reverse inhibition of either spore outgrowth or vegetative growth and higher concentrations of both chemicals were inhibitory to outgrowth.

Since the spore escapes fully from chloroquine inhibition as the germ-cell emerges from the spore coat, it is possible that the drug prevents emergence from the coat, and so development. The vegetative cell, having no coat, would not be restricted by this mechanism. By removing the coat with alkaline - SDS - dithiothreitol reagent spores become sensitive to lysozyme (Aronson & Fitz-James, 1968) and so the coat is either removed or weakened. Such 'stripped' spores were still sensitive to chloroquine during outgrowth in the presence of 20% sucrose. ~~therefore~~

~~chloroquine sensitivity is not related to the ability to break the coat open at emergence (data not shown)~~. Furthermore escape from the drug began before emergence of the germ-cell (Fig. 13) and although it can be argued that the coat becomes weaker as outgrowth proceeds the experiments with 'stripped' spores suggest the strength of the coat is immaterial to chloroquine inhibition.

The inhibition of both DNA and RNA polymerase activities by chloroquine was found to be reversed by adding divalent cations (Cohen & Yielding, 1965a; Whichard et al., 1972). Addition of various ions also reversed the inhibitory effects of chloroquine in both outgrowing spores and vegetative cells. Figures 14 and 15 show a representative reversal of chloroquine inhibition by $MnCl_2$ during spore outgrowth and vegetative growth. Ions reversed chloroquine inhibition if added before or after growth was prevented by the drug. Other divalent cations, Mg^{2+} or Ca^{2+}, also reversed this inhibition as either chloride or sulphate salts (Table 8). NaCl or KCl at higher concentrations relieved inhibition to a lesser extent (Table 8). The inhibitory effect of chloroquine on sporulation was not relieved by any concentration of Mn^{2+}, indicating the existence of a difference in the inhibitory mode of action between outgrowing and sporulating cells or penetration of Mn^{2+}.

The reversal of chloroquine inhibition in the presence of ions may be due to either an interaction between the chloroquine and ions or competition for binding sites at the drug's target. Cohen and Yielding (1965a) found a competative relationship between Mg^{2+} and chloroquine inhibition of DNA polymerase, consistent with the interpretation that chloroquine was competing for the DNA substrate. Alternatively chloroquine

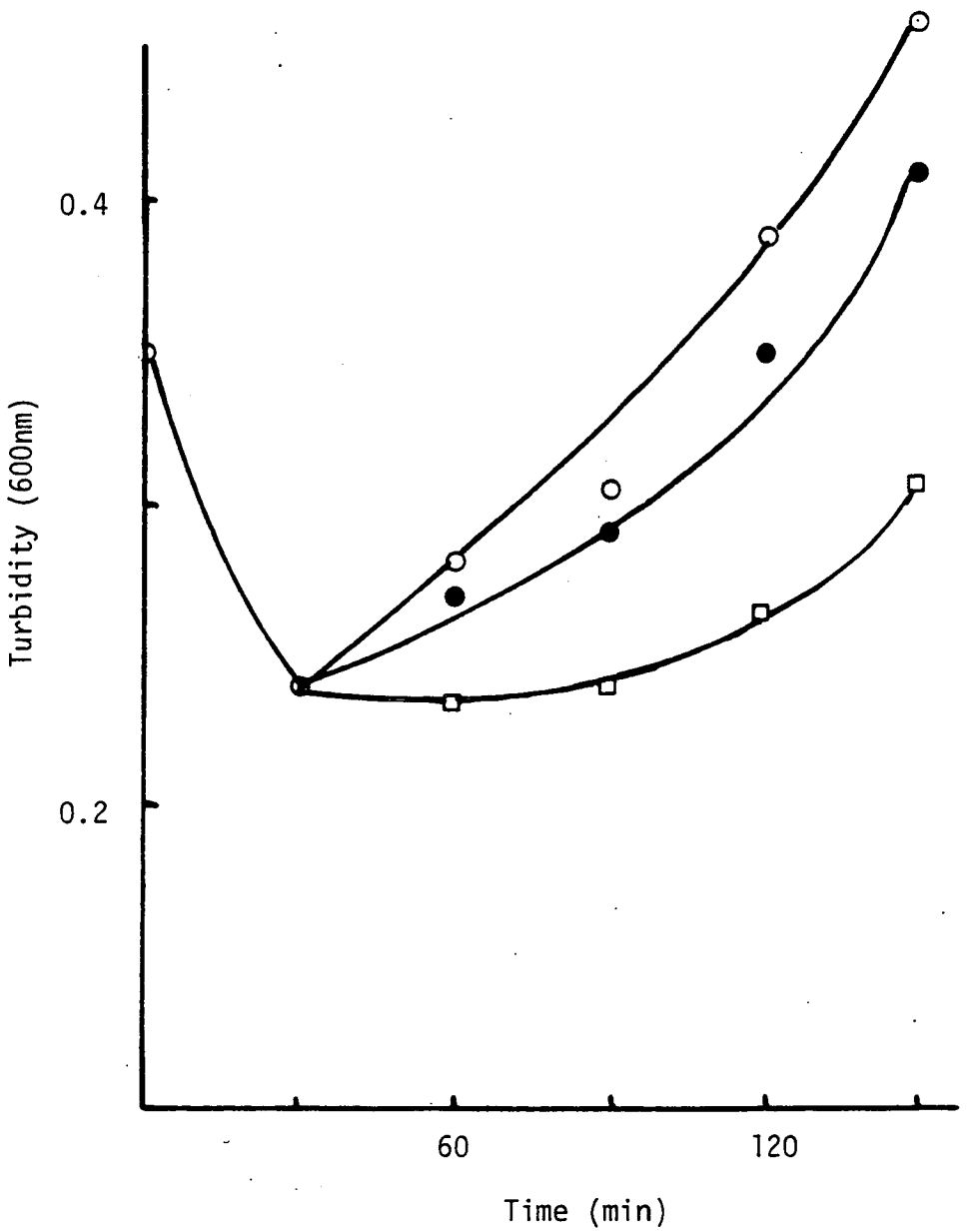


Figure 14. Reversal by $MnCl_2$ of chloroquine inhibition of spore outgrowth. Outgrowth was measured as described earlier. Chloroquine (500ug ml^{-1}) was added at zero time: (○), no chloroquine; (●), 0.25mM $MnCl_2$ added at zero time; (□), 0.25mM $MnCl_2$ added at 90 min.

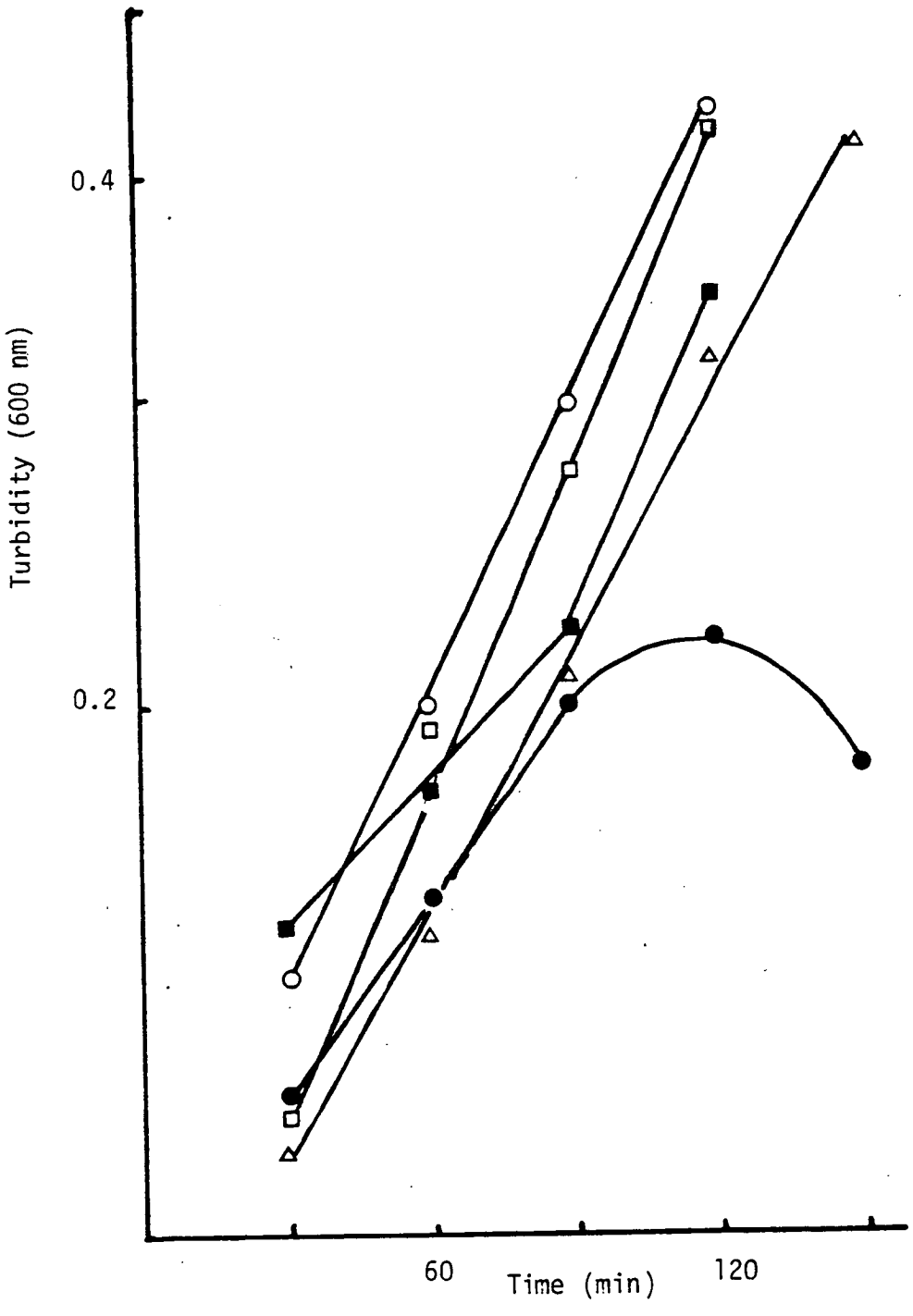


Figure 15. Reversal of inhibition of vegetative growth by $MnCl_2$. Vegetative growth was measured as described earlier. Chloroquine (5mg ml^{-1}) was added at 30 min: (○), no chloroquine; (●) 5mg ml^{-1} chloroquine; (□), 2.5mM $MnCl_2$ added at 30 min; (■) 2.5mM $MnCl_2$ added at 90 min; (△) 0.25mM $MnCl_2$ added at 30 min.

Table 8

Concentration of ions relieving chloroquine inhibition

concentration relieving inhibition mM

Salt	Outgrowth[a]	Vegetative growth[b]
$MnCl_2$	0.25	0.25
$MnSO_4$	0.25	nd
$MgCl_2$	0.25	0.25
$MgSO_4$	0.25	nd
$CaCl_2$	0.25	0.25
KCl	>100	200
NaCl	>100	200

Outgrowth and vegetative growth were measured as described in the methods section. Ions were added to the growth medium, NB and minimal medium.

(a) 500 $ugml^{-1}$ (1mM) chloroquine added.

(b) 5mg ml^{-1} (10mM) chloroquine added.

n d Not done

may be chelating ions, although Cohen and Yielding (1965a) found no spectrophotometric or potentiometric evidence for this.

Table 9 shows the activity of glucose-6-phosphate dehydrogenase (GPDH. in the presence of chloroquine, this enzyme has no requirement for Mg^{2+} but its activity is modulated by different concentrations of Mg^{2+} (Biochemica information 1., Boehringer Mannheim 1973). If chloroquine chelates Mg^{2+} one would expect it to alter the enzyme activity. Chloroquine did not alter the enzyme activity although EDTA, a known chelator, increased activity by 27%. The chloroquine inhibition of GPDH reported by Cotton and Sutorius (1971) at low $NADP^+$ levels was not evident at the higher $NADP^+$ levels used here. The inhibitory effect of chloroquine during spore outgrowth was not reversed by $NADP^+$ or NADH (0.5-2mM) added to the medium, although it is doubtful if either molecule can enter the cell, and the possibility that chloroquine prevents reduction of $NADP^+$ or NAD^+ or competes with the co-enzymes cannot be discounted. Addition of ATP (1-2mM) togrowth medium did not reverse chloroquine inhibition but again ATP is unlikely to penetrate the cell membrane.

Chloroquine as an intercalating agent

The prime target for chloroquine appears to be related to its interaction with DNA, probably intercalating with the macromolecule (Ciak & Hahn, 1966; Waring, 1970). If chloroquine inhibits spore outgrowth by intercalating into DNA it might be expected to act in an additive or synergystic manner with other intercalating molecules. This hypothesis was tested using a known intercalating drug, ethidium bromide. Table 10 shows that the relationship between ethidium bromide and chloroquine

Effect of chloroquine on the activity of glucose-6-phosphate dehydrogenase

Chloroquine 500 ug ml (1mM)	EDTA 1mM	$MgCl_2$ 125 uM	$MgCl_2$ 250 uM	Observed enzymeactivity $\Delta E\ min^{-1}$
0	0	0	0	13.2
+	0	0	0	13.0
0	+	0	0	16.8
0	0	+	0	18.0
0	0	0	+	17.6
+	0	+	0	17.4
+	0	0	+	17.4
+	+	0	0	17.0

Enzyme activity was measured by following the reduction of $NADP^+$ at 366nm to avoid interference by chloroquine absorbance (E) at 340nM.

was of a synergystic nature, mixtures of both drugs each at concentrations below that causing complete inhibition, did together completely inhibit outgrowth. It is difficult to interpret with certainty the results of this type of study. For example if the two drugs were interacting with DNA in precisely the same way then one might expect an additive effect of using a mixture. On the other hand if the two drugs intercalate with a different specificity (e.g. some authors have suggested chloroquine may act preferentially on G/C rich regions: Sternglanz et al. 1969). Then a combination of the two may lead to a synergystic response. However, the observed synergism can be taken to indicate that chloroquine (at 500 ug. ml^{-1}) is exerting its influence on spore outgrowth by interacting with DNA or its metabolism in some way. This possibility can be tested more directly by considering the various functions, e.g. DNA replication, that may be affected.

Chloroquine inhibition and DNA synthesis

The onset of chloroquine resistance during outgrowth correlates with the beginning of DNA replication (Chapter 5, Fig. 22). DNA replication in bacteria is however, a complicated process requiring an initiation step in addition to those concerned with synthesis. It is unlikely that the main effect of chloroquine is on DNA synthesis per se, since outgrowth of B. cereus and B. subtilis spores is independent of DNA synthesis when nalidixic acid (an inhibitor of DNA gyrase) is used to prevent DNA synthesis (Dawes & Halvorson, 1972; Ginsberg & Keynan, 1978). In fact, while chloroquine may be intercalating with DNA its inhibitory effect is not apparently dependent on DNA synthesis either since chloroquine (500 ug. ml^{-1}) inhibited outgrowth in which replication was

Table 10

Synergystic inhibition of spore outgrowth by chloroquine and ethidium bromide

Inhibitor $ug.ml^{-1}$	Percentage of original turbidity*	Percentage inhibition
None	150	0
Ethidium bromide (3)	141	14
Chloroquine (100)	126	34
Ethidium bromide chloroquine (100)	78	100
Chloroquine (500)	77	100
Ethidium bromide (5)	77	100

Outgrowth was measured as described in the methods section.

Inhibitors were added to the growth medium (NB) at 0 time.

* % increase in turbidity (600nm) from 0 time to septation (150 min).

prevented by the addition of nalidixic acid (Fig. 16). Furthermore, DNA synthesis does not seem to be necessary for the development of resistance to chloroquine during outgrowth, since outgrowth beyond 100 min occurred normally in the presence of chloroquine at 500 ug.ml^{-1} and nalidixic acid.

Effect of chloroquine on B. subtilis ts-134

The above results have ruled out the possibility that DNA synthesis has any involvement in the site of action of chloroquine or in the onset of resistance to chloroquine during outgrowth. However nalidixic acid does not inhibit the intiation steps in replication and there are a number of limits on using nalidixic acid since it does not completely inhibit DNA synthesis and as an inhibitor of DNA gyrase activity can influence transcription from certain gene promoters (Ginsberg & Keynan, 1978; Sanzey, 1979). Therefore the effect of chloroquine inhibition on DNA synthesis was tested using spores of ts-134, a mutant of B. subtilis 168, temperature-sensitive for initiation of DNA replication (Mendelson & Gross, 1967). Using this mutant one can test chloroquine inhibition in the absence of DNA... replication and initiation of DNA replication, also to test for any effect on the development of resistance to chloroquine of specifically inhibiting the initiation of DNA replication.

Spores were grown out at 48^{o}C to minimise the initiation of DNA replication observed in spores of this mutant of 45^{o}C (Callister et al., 1977). Spores of ts-134 at 48^{o}C grew out normally* but chloroquine prevented this elongation (data not shown). Therefore it is unlikely that chloroquine acts during spore outgrowth on either the

*also see Callister et al. (1977).

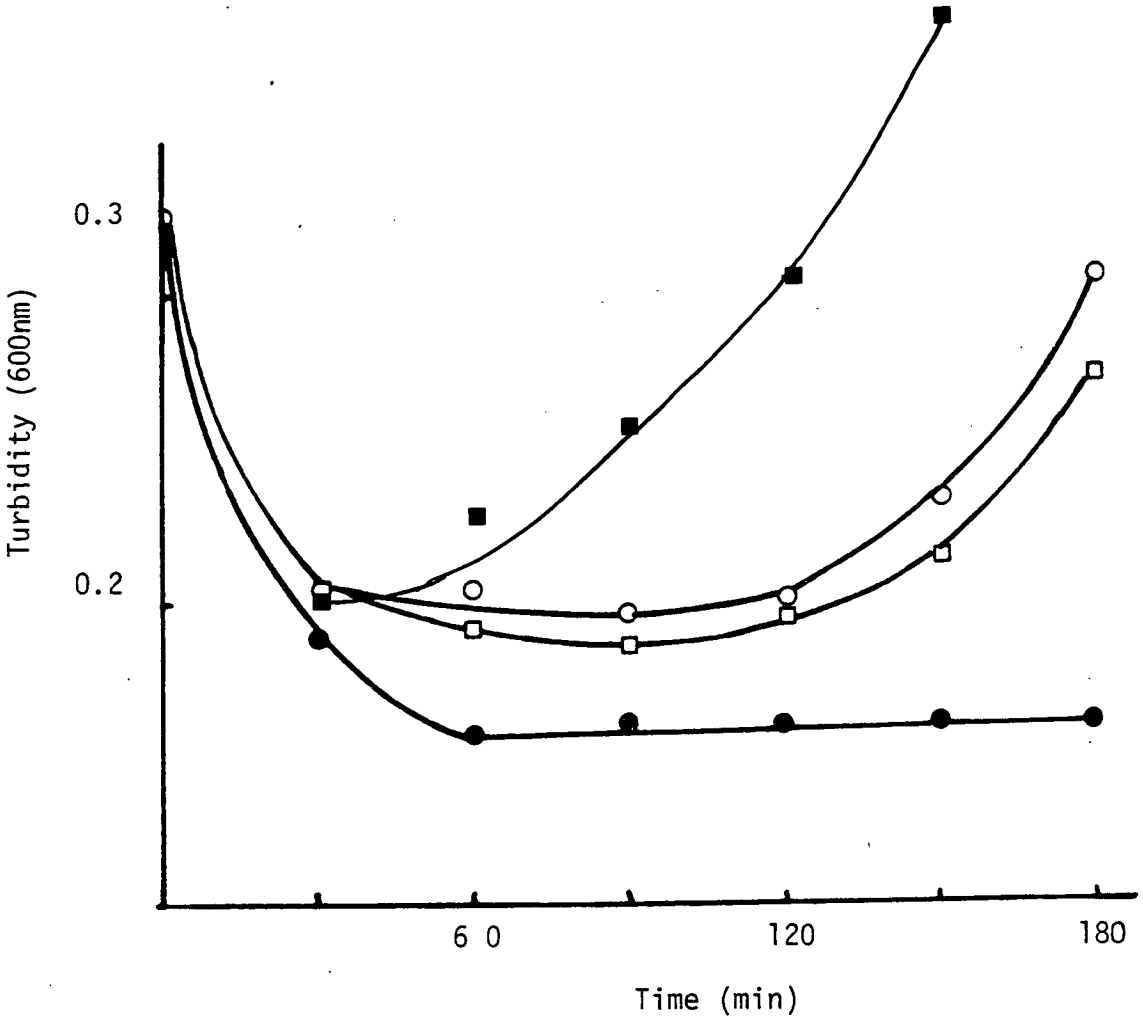


Figure 16. The effect of chloroquine on spore outgrowth in the presence of nalidixic acid. Spore outgrowth was measured as described earlier. Nalidixic acid (10 ug ml^{-1}) was added at zero time: (○), nalidixic acid; (●) nalidixic acid and chloroquine (500ug ml^{-1}) added at zero time; (□), nalidixic acid and chloroquine (500ug ml^{-1}) added at 100 min; (■), no nalidixic aci

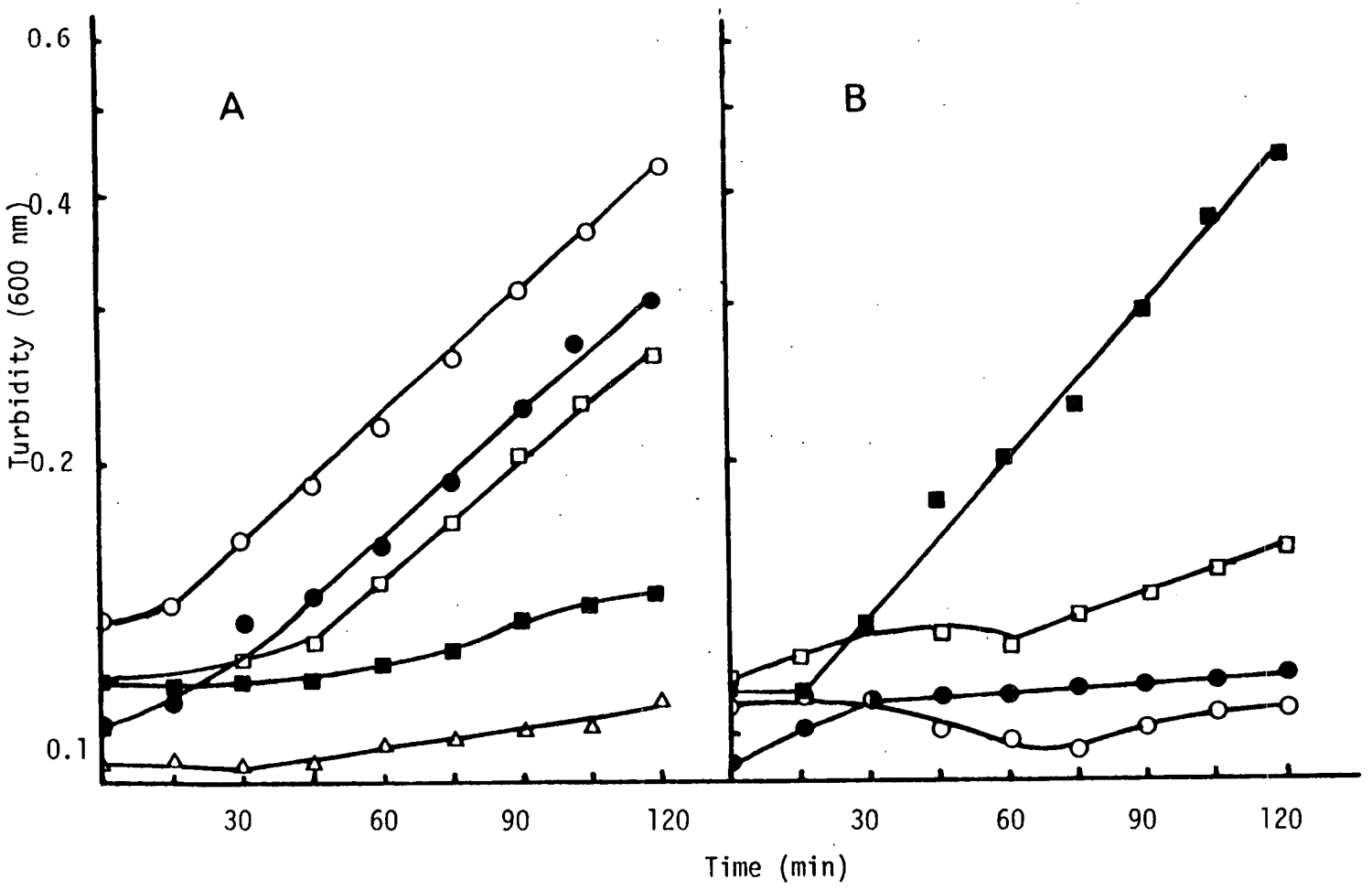
initiation of DNA replication or further synthesis of DNA.

In doing the control experiment using vegetative cells of ts-134, however, an interesting result was obtained. If vegetative cells of this mutant were held at 45°C for 1 hour, they became sensitive to a lower level of chloroquine (500 ug ml^{-1}) on return to the permissive temperature of 30°C. Holding ts-134 at 45°C for 1 hour did not affect its ability to resume vegetative growth on return to 30°C (a lag of 15 min) in the absence of chloroquine (Fig. 17). This inhibition of vegetative cells of ts-134 by chloroquine at 500 ug ml^{-1} was apparent after 15 min at the restrictive temperature. On returning the cells to the permissive temperature (30°C) after 1 hour at 45°C resistance to chloroquine was observed to develop at about 45 min (Fig. 17). The mutant showed normal resistance to chloroquine at 30°C, wild type cells showed slightly increased sensitivity at 45°C (Fig. 18).

Chloroquine inhibits growth in the absence of DNA synthesis, if chloroquine is intercalating with DNA it may be preventing transcription. It is possible to reconcile inhibition of vegetative cells of ts-134 after incubation at 45°C, preferential outgrowth of spores and inhibition of sporulation by considering the state of the chromosome in each of the cases. Dormant spores, sporulating cells and ts-134 after 1 hour at 45°C all have a complete, non-replicating chromosome. (Hitchins, 1978; Mendelson & Gross, 1967), chloroquine may inhibit transcription specifically from such a chromosome. If this is the case, by adding nalidixic acid at zero time to prevent replication of the chromosome during spore outgrowth there should be no escape from 500 ug ml^{-1} chloroquine inhibition, however, outgrowing spores in the presence of nalidixic acid became less sensitive to chloroquine as normal (Fig. 16)

LEGEND

Figure 17. Timing of chloroquine inhibition of ts-134 vegetative cells at the non-permissive temperature (45°C). Vegetative cell growth was measured as described previously. (A) ts-134 was grown at 45°C in NB. Samples were removed at the times indicated and incubated at 30°C. Chloroquine (500 ug.ml^{-1}) was added at zero time. (A) Time at 45°C: (○), 0 min; (●), 15 min; (□), 30 min; (■) 45 min; (△), 60 min. (B) ts-134 incubated at 30°C after 1 hour at 45°C: (○), 15 min; (●), 30 min; (□), 45 min; (■), omin no chloroquine.


A
B
Turbidity (600 nm)
0.6
0.4
0.2
0.1
30
60
90
120
30
60
90
120
Time (min)

Spores of ts-134, incubated at 48°C, however, did not show any decreased sensitivity to chloroquine. It is possible that chloroquine inhibits transcription from a chromosome that has not been initiated to undergo DNA replication. This would explain why outgrowing spores of ts-134 at 48°C did not escape acquisition of resistance to chloroquine may depend on some event related to initiation of replication e.g. a membrane function or an ion fluctuation. The ts-134 mutant has been shown to accumulate a membrane protein on prolonged exposure to the restrictive temperature (Harmon & Taber, 1977). Addition of $MnCl_2$ or $MgCl_2$ failed to relieve the inhibition of ts-134 vegetative cells by 500 ug ml^{-1} chloroquine after 1 hour at 45°C and this may indicate that the mutant is altered in its ability to accumulate ions. An alternative explanation is that the inhibition of mutant vegetative cells and wild type spores by 500 ug ml^{-1} chloroquine is unrelated.

Further evidence against preferential inhibition of transcription from a completed chromosome was provided by studying slowly growing vegetative cells. Cells were grown in minimal salts medium with glucose replaced by glutamate, the generation time in this medium was about 420 min, and for about one third of every cell division cycle the organism should have a completed chromosome (Lewin, 1974). Although chloroquine inhibition was increased considerably, complete inhibition would be expected if cells with completed chromosomes were inhibited preferentially (Fig. 19). However, it is possible that chloroquine slowed down DNA replication so that it occupied a complete cycle, although this does not appear to be the case in cells grown with glucose (Chapter 5, Fig. 24). Similarly, it is possible (but unlikely) that vegetative cells could continue growth without much new transcription until DNA replication re-starts, whereas germinated

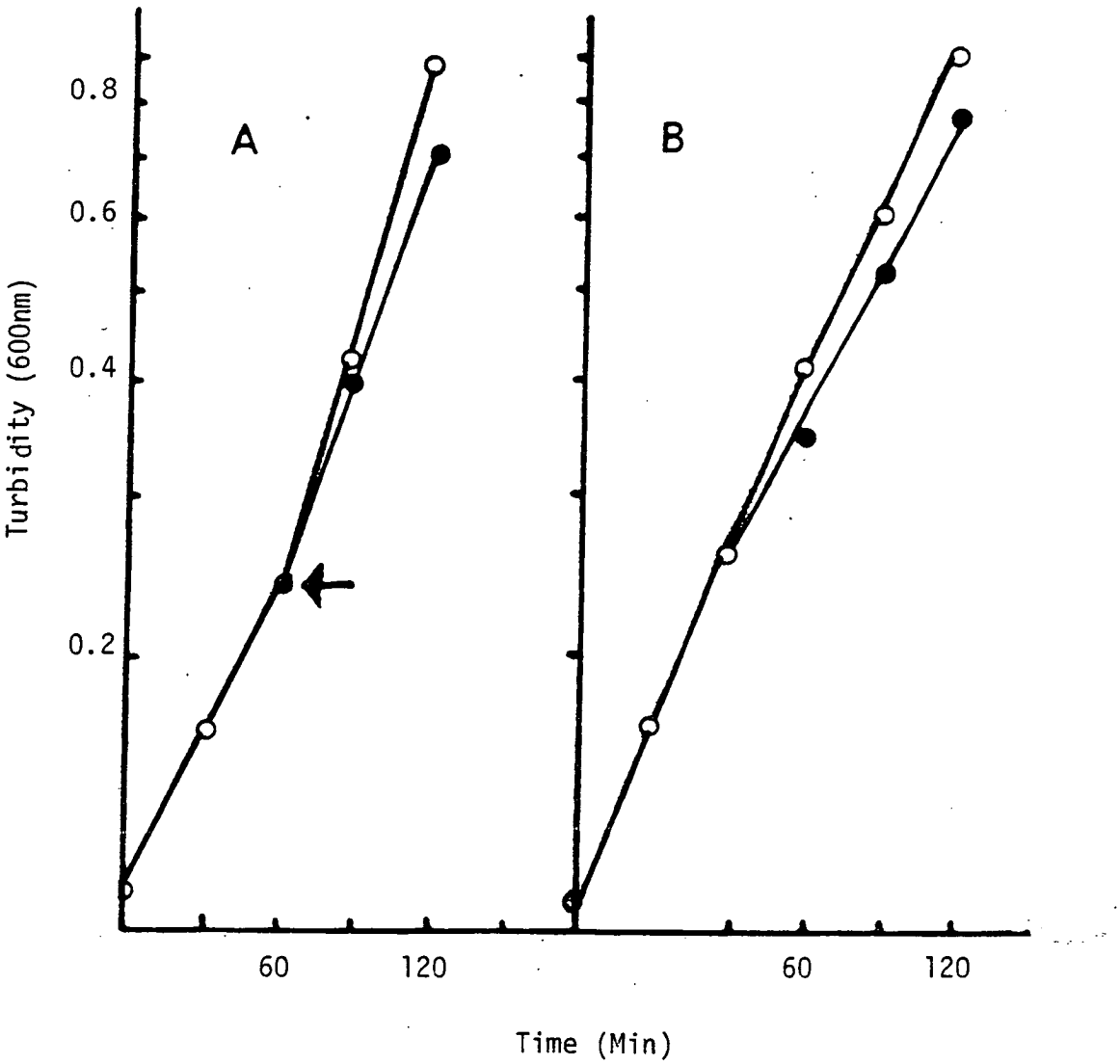


Figure 18. Effect of chloroquine on wt cells at 45°C and ts-134 cells at 30°C. Vegetative cell growth was measured as described previously. (A) wt cells were incubated at 30°C then shifted to 45°C at the time indicated by the arrow. Chloroquine was added at 60 min: (O), no chloroquine; (●), chloroquine (500ug. ml^{-1}) (B) ts-134 cells grown at 30°C: (O), no chloroquine; (●), 500 ug ml^{-1} chloroquine added at 60 min.

spores which do not have a full complement of vegetative proteins would not have sufficient reserves to do so. A further factor which may influence transcription from a completed chromosome is the observation that in spores the chromosome is diffuse and unbound to the membrane, during outgrowth the chromosome condenses and binds to the membrane (Ryter, 1968; Ryter et al., 1968). It is possible that the chromosome is more susceptable to chloroquine inhibition in the diffuse unbound state, although this would not account for inhibition of ts-134 growth at the non-permissive temperature because the chromosome is still bound to the membrane (Harmon & Taber, 1977).

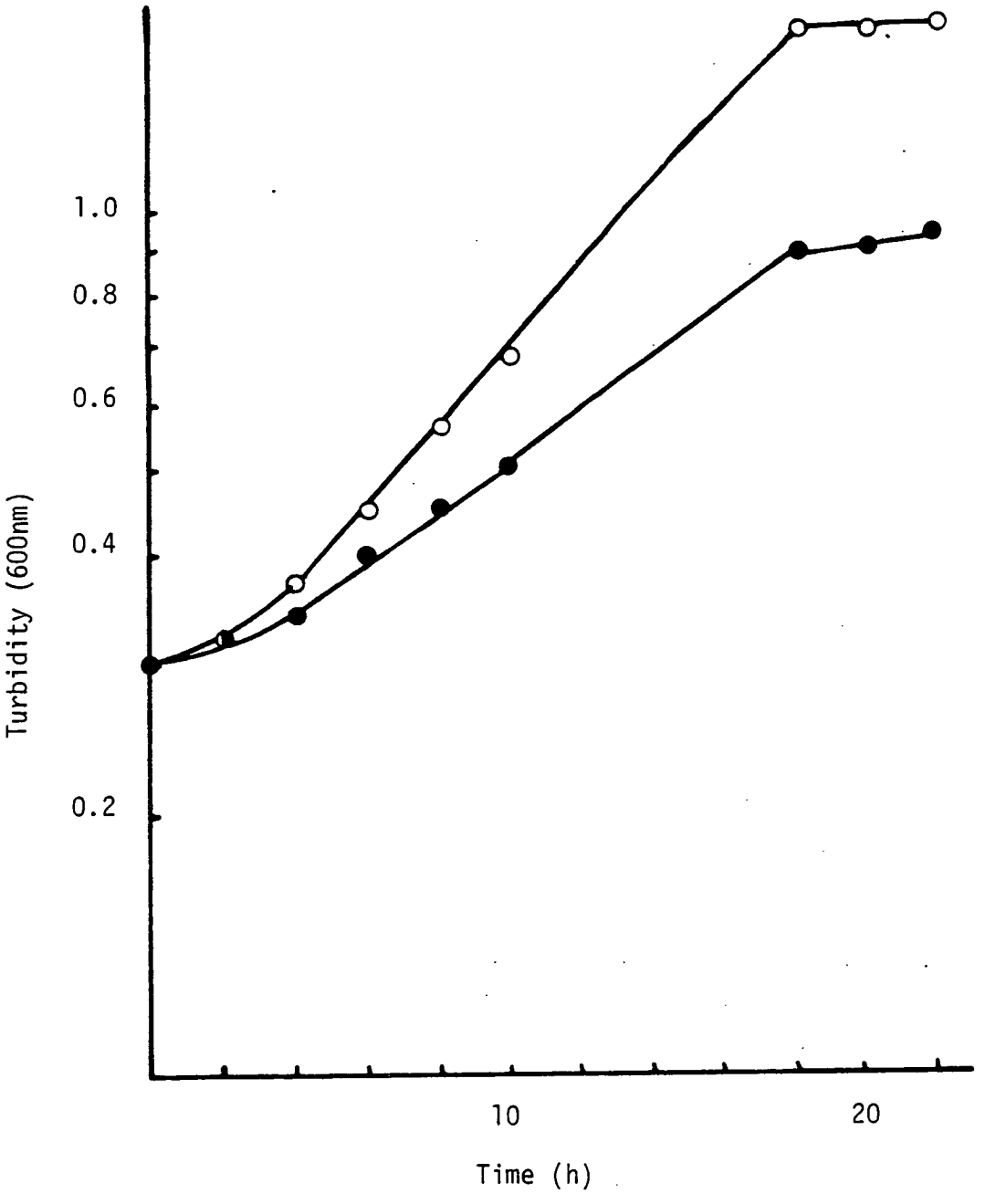


Figure 19. Effect of chloroquine on slow growing vegetative cells. Cell growth was measured as described earlier. Cells were grown at 30°C in minimal medium with 0.5% glutamate as carbon source (○), no chloroquine; (●), chloroquine (500 ug ml^{-1}) added at zero time.

Conclusions

In this chapter several of the reported actions of chloroquine have been investigated and the preferential inhibition of spore outgrowth more fully characterized.

The lowest concentration of chloroquine (500ug ml^{-1}) which prevented spore outgrowth was not sporicidal in agreement with the findings of McDonald (1967). However, higher concentrations of chloroquine (5mg ml^{-1}) killed vegetative cells, which lysed after prolonged exposure to the drug (chapter 3). It is unlikely that the bactericidal action of 5mg ml^{-1} chloroquine on vegetative cells is due to a direct attack on the cell membrane, although a molecule with both positively charged polar nitrogen group and a hydrophobic region, like chloroquine, can act as a cationic detergent (Fig. 11; Harold, 1970). If chloroquine acts by disorganising the cell membrane, one would not expect the extended period of cell elongation before cessation of growth (Fig. 10) because the cell would become leaky and lose essential metabolites early after addition of the drug so preventing biosynthetic events. The same argument can be applied to the action of 500ug ml^{-1} chloroquine on spore outgrowth, if chloroquine were disrupting the membrane and allowing leakage of metabolites it is unlikely that outgrowth would be able to resume on removal of the drug, particularly in the absence of an extensive biosynthetic capacity during early spore outgrowth (Section 2.1, Chapter 1).

The observation that chloroquine inhibition is independent of medium composition shows that chloroquine has no obvious structural anology with an important metabolite or the inhibition would be reversed,

particularly in a very rich medium such as penassay broth which contains many important vitamins etc. It is more difficult to judge the significance of the failure of $NADP^+$, NADH, thiol-reagents and ATP to reverse chloroquine inhibition. Chloroquine has been shown to inhibit thiol enzymes and act competetively with $NADP^+$ in enzyme reactions (Fiddick & Heath, 1967; Cotton & Sutorius, 1971). While one might expect chloroquine inhibition to be reversed by thiol-reagents and $NADP^+$, if either of these were related to its mode of action a number of factors could influence the outcome of adding these molecules. For $NADP^+$ and NADH it is unlikely that these molecules can enter the cell and unless they compete for an uptake system it is unlikely they could reverse inhibition externally. The thiol-reagents, cysteine and dithiothreitol may again not be able to enter the cell although the observation that they inhibit spore outgrowth and vegetative cell growth may indicate some uptake does occur and if chloroquine were binding to thiol groups extracellular -SH groups might be expected to detoxify the drug to some extent, this effect was not observed.

The function related to chloroquine sensitivity of outgrowing spores changed after about 50 min of germination and outgrowth in NB, concurrent with germ-cell emergence, and became resistant to 500ug ml^{-1} chloroquine. It is unlikely that chloroquine prevents emergence from the spore coat and so further development because germinated spores with weakened spore coats are still sensitive to chloroquine and partial escape from chloroquine inhibition begins before the spore coat breaks.

Chloroquine acts in concert with ethidium bromide to prevent spore outgrowth and therefore may act by intercalating with DNA. Further evidence for an action on DNA was provided by the experiments that

show divalent cations and to a lesser extent monovalent cations reversed chloroquine inhibition of both outgrowing spores and vegetative cells and in the absence of any evidence for chloroquine chelating ions, it is likely that the ions compete with chloroquine for binding sites on the DNA (Cohen & Yielding, 1965a). Further evidence for this was provided by Cohen & Yielding (1965b) who have shown that the raising of the melting point of double stranded DNA caused by chloroquine was reversed by ions. If chloroquine was chelating ions one would not expect the ions to reverse chloroquine inhibition after the drug has prevented growth unless the drug had a greater affinity for the ions than for the target, if this were so the changes in the absorbance spectrum of chloroquine observed in the presence of DNA would be seen in the presence of ions. However there is no change in the absorbance spectrum of chloroquine with Mg^{++} or Mn^{++} present (data not shown). Furthermore, there is no evidence of chelation of Mg^{++} ions by chloroquine in an enzyme assay sensitive to chelating agents.

If chloroquine were binding to DNA as its intracellular target, there are a number of ways that it could inhibit further cell growth. It may function to inhibit either (i) chromosome replication (ii) chromosome segregation or its attachment to membranes (iii) transcription or (iv) DNA repair. Chromosome replication is a complicated process involving an initiation step that requires protein synthesis, and subsequently the involvement of a large number of gene products in the synthesis steps (Lewin, 1974). Net DNA synthesis *per se* is not essential for outgrowth to occur up to, but not including, the stage of cell septation, as shown by the use of the DNA gyrase inhibitor, nalidixic acid and mutants temperature - sensitive for DNA synthesis (Ginsberg & Keynan, 1978). The results presented here using chloroquine alone and

mixtures of chloroquine and nalidixic acid clearly indicate that the primary effect of chloroquine in preventing outgrowth is not on DNA synthesis. Similarly, the fact that the initiation defective mutant ts 134 can complete outgrowth at the restrictive temperature indicates that the primary site of chloroquine action is not likely to be at the initiation of replication since chloroquine prevents outgrowth when added to susceptible spores. Repair-like DNA synthesis does occur during outgrowth of spores of certain Bacillus spp. but it is unlikely that this is significant to spore outgrowth (Section 2.4 , Chapter 1). Nevertheless chloroquine prevents repair of U V-damage in E. coli (Yielding et al., 1970) and was found to prevent DNA repair of UV-damage in vegetative cells of B. subtilis (unpublished results) and it is possible this action contributes to the inhibitory effect of chloroquine. No conclusions can be made here about the effect of chloroquine on transcription.

Chloroquine can inhibit growth without apparently interfering with membrane functions and the biosynthesis of precursors of macromolecular synthesis, furthermore it may act by intercalating with DNA. It is probable from this that chloroquine is preventing a polymerization step in macromolecular synthesis although inhibition of DNA synthesis or of the initiation of DNA replication are not its direct site of action during outgrowth of spores.

The effect of chloroquine on macromolecular synthesis is examined more fully in the next chapter.

CHAPTER 5

Macromolecular synthesis in the presence of chloroquine

Introduction

In the previous chapter it was concluded that the target for chloroquine action in *B. subtilis* is DNA or some aspect of its metabolism, and that the drug probably acts by binding to DNA.

Since chloroquine does not appear to act on outgrowing spores by inhibiting the initiation of DNA replication or DNA synthesis, and DNA repair is not important during outgrowth (see Section 2.4), the most likely candidate for the immediate target is transcription. Chloroquine does affect RNA synthesis as well as DNA synthesis in *Plasmodium knowlesi* and *B. megaterium* (Polet & Barr, 1968; Ciak & Hahn, 1966). If RNA synthesis were inhibited then protein synthesis would also be affected, and also DNA synthesis during outgrowth since initiation of replication depends on protein synthesis.

To test this hypothesis, the effect of chloroquine on synthesis of all three classes of macromolecule has been examined, in ourgrowing spores (using the lower concentration of chloroquine) and vegetative cells (a both lower and higher concentrations).

Effect of chloroquine on macromolecular synthesis during outgrowth

RNA is the first macromolecule synthesized during outgrowth, and Figure 20 shows the effect of chloroquine on the incorporation of [^{3}H]-uracil into RNA of outgrowing spores. Spores incubated in the absence of chloroquine demonstrated the expected phases of uracil incorporation into RNA; early synthesis in the first 90 min preceded a period of no net incorporation. Coincident with cell elongation beginning at 150 min, RNA synthesis resumed at an increasing rate. Chloroquine (500ug ml^{-1}) prevented the later large scale RNA synthesis but there was some slight incorporation. When chloroquine was added after the escape time (150 min), RNA synthesis followed the same pattern as it did in cells lacking the drug although the rate of synthesis was 30% less. A non-inhibitory level of chloroquine (100ug ml^{-1}) added at zero time led to a very similar level of incorporation as was found for 500ug ml^{-1} of the drug added at the escape time; normal incorporation was obtained if 100ug ml^{-1} chloroquine was added at 150 min.

The incorporation of [^{14}C]-amino-acids into protein is shown in Figure 21, with the same basic pattern as was seen for RNA synthesis, although protein synthesis began after RNA synthesis. Again chloroquine prevented large scale synthesis when added at zero time, and incorporation was reduced by 50% when the drug was added at 150 min. The same level of inhibition (50%) was observed when 100ug ml^{-1} chloroquine was added at zero time, there was no effect if 100ug ml^{-1} of the drug was added at the escape time.

[^{3}H]-Thymidine incorporation into DNA followed the same pattern

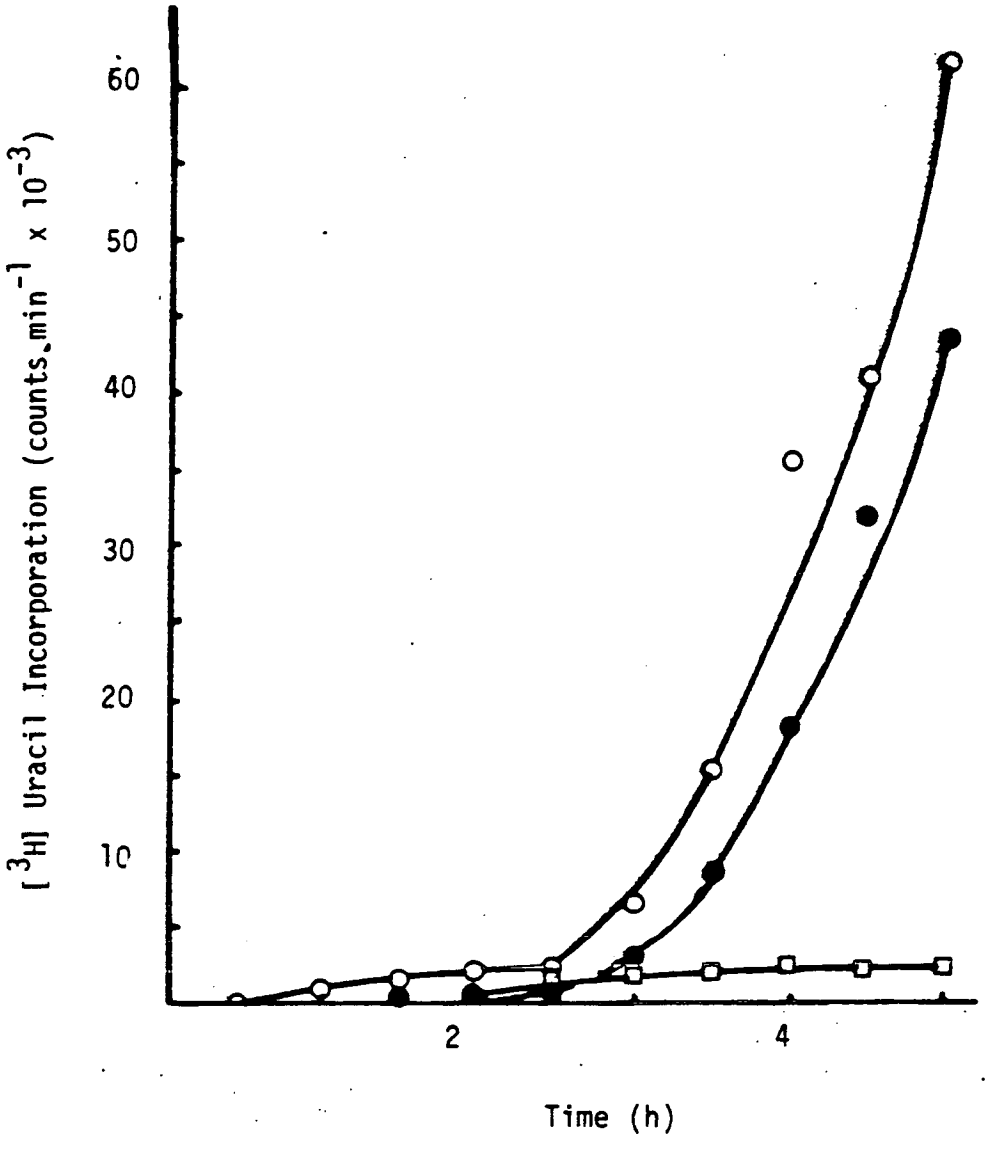


Figure 20. Effect of chloroquine on RNA synthesis during spore outgrowth. Spores (10^8) were inoculated into 10ml of the synthetic outgrowth medium of Kennett and Sueoka (1971), supplemented as described in the methods. RNA synthesis was measures as the uptake of [^{3}H]-uracil into TCA-insoluble material: (○), no chloroquine; (●), chloroquine (500ug ml^{-1}) added at 150 min. (□), chloroquine added at zero time.

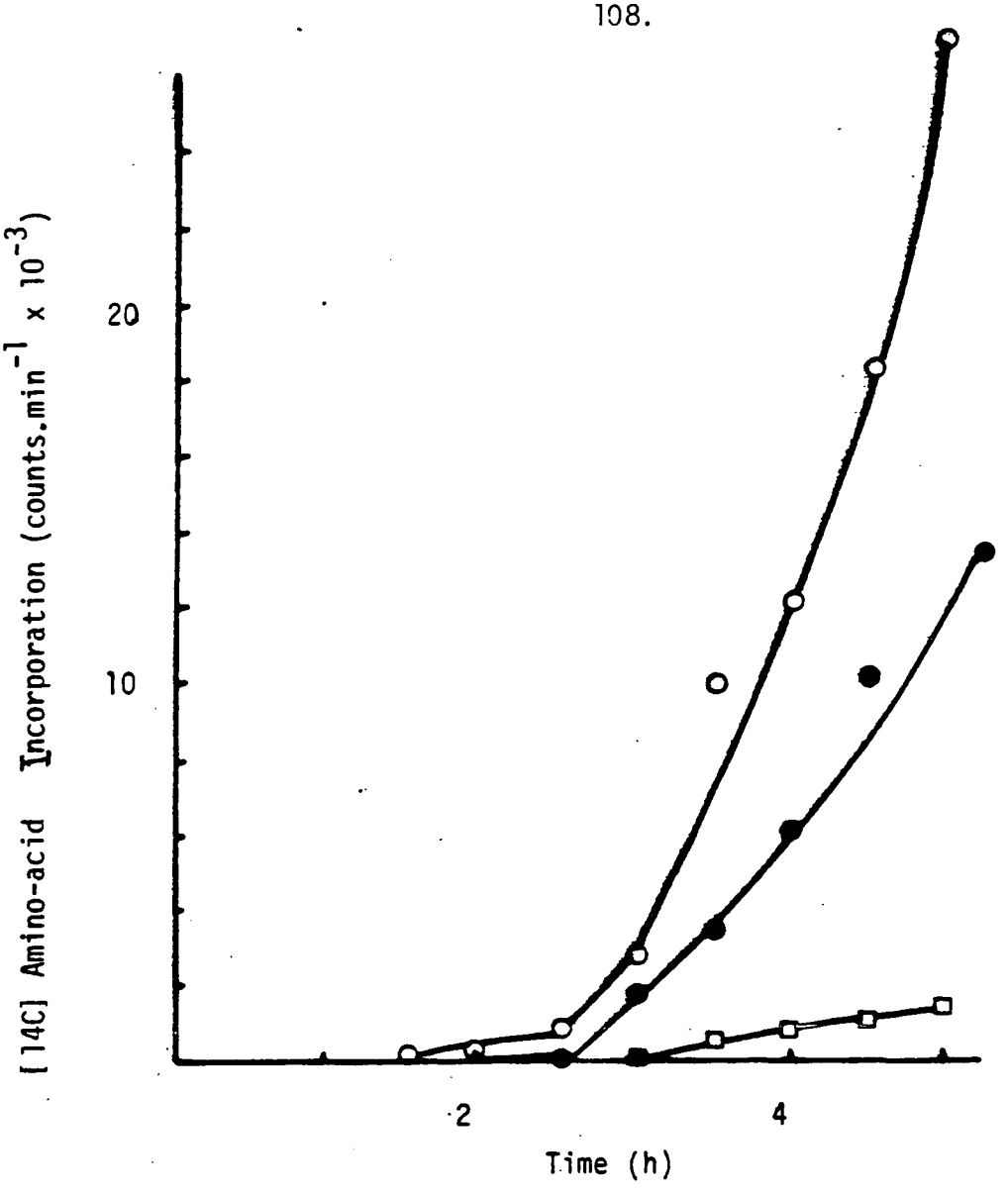


Figure 21. Effect of chloroquine on protein synthesis during spore outgrowth. Spores (10^8) were inoculated into 10ml of the synthetic outgrowth medium of Kennett and Sueoka (1971), supplemented as described in the methods. Protein synthesis was measured by the incorporation of [^{14}C]-amino-acids into TCA-insoluble material: (○), no chloroquine; (□). chloroquine (500 ug.ml^{-1}) added at zero time; (●), chloroquine (500ug.ml^{-1}) added at 150 min.

as RNA and protein synthesis. There was a brief 30 min period of synthesis, followed by 150 min in which there was no net incorporation and at the third phase of outgrowth, when cell elongation commenced, large scale synthesis of DNA was seen, continuing towards septation (Fig. 22). Chloroquine added from germination prevented only the later phase of DNA synthesis commencing at cell elongation, the early synthesis was unaffected by the drug (Fig. 22). When chloroquine (500ug ml^{-1}) was added at the escape time only about a 20% reduction in incorporation of label was seen by cell septation (Fig. 22).

From these results if chloroquine does interact with DNA it seems likely that the effect of chloroquine on outgrowth is to inhibit synthesis of some class of RNA that is required in promoting the active phase of macromolecular synthesis as the germinated and swollen spore begins to grow. This would lead to inhibition of DNA as well as protein synthesis, since during spore outgrowth net DNA synthesis depends on prior protein synthesis which in turn depends on the synthesis of all major species of RNA. However, it is difficult to distinguish between a prior effect of chloroquine on RNA synthesis or on protein synthesis due to the strict interdependence of these two processes. However, it should be possible to determine which of these two is affected first in a vegetatively growing cell, sincethe complications of low initial rates of synthesis, and of an ordered programme of events are avoided. It is possible that chloroquine inhibits spore outgrowth and vegetative growth in different ways, nonetheless it is worth determining what takes place in the vegetative cell, since the differential effect may not arise from differences in the target.

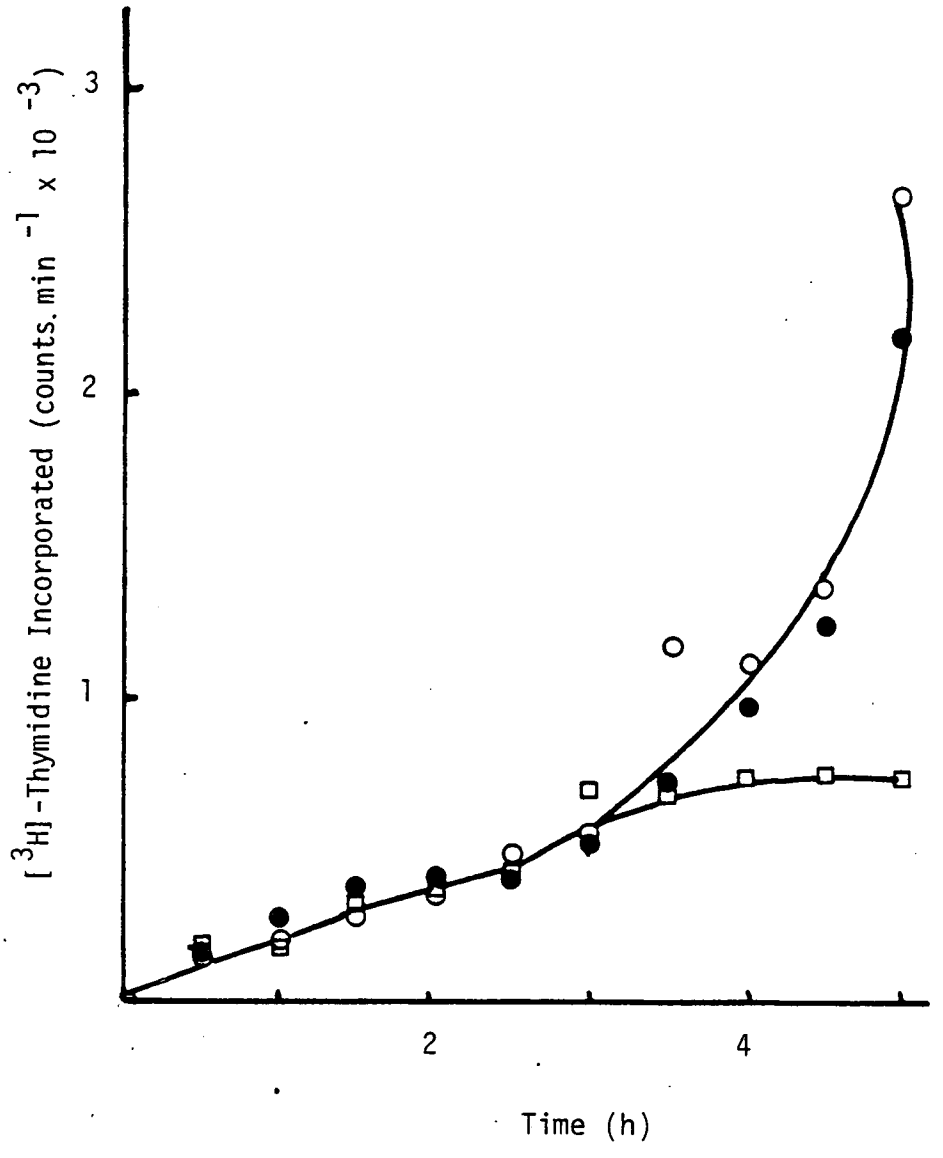


Figure 22. Effect of chloroquine on DNA synthesis during spore outgrowth. Spores (10^8) were inoculated into 10ml of the synthetic outgrowth medium of Kennett and Sueoka (1971), supplemented as described in the methods. DNA synthesis was measured as the uptake of [^{3}H]-thymidine into TCA-insoluble material: (○), no chloroquine; (□), chloroquine (500ug.ml^{-1}) added at zero time; (●), chloroquine (500ug ml^{-1}) added at 150 min.

Effect of chloroquine on macromolecular synthesis during vegetative growth

In vegetative cells, chloroquine (500ug.ml^{-1}) immediately inhibited the rate of uracil incorporation by 30%, a higher concentration of the drug (5mg ml^{-1}) decreased the rate of uracil incorporation immediately and prevented it completely within 30 min, thereafter label was lost from the RNA (Fig. 23A). However, protein synthesis was inhibited only slightly in the first 45 min that chloroquine (500ug ml^{-1}) was present. At a concentration of 5mg ml^{-1} it reduced the rate of protein synthesis to 30% of the control value, but synthesis did continue for 45 min before degradation became apparent (Fig. 23B). In other words the effect of chloroquine (5mg.ml^{-1}) was more rapid and more marked on RNA synthesis than on protein synthesis.

The results obtained for the incorporation of [^{3}H] - thymidine into DNA were not reproducible and therefore DNA was measured chemically by the diphenylamine assay of Burton (1956) which is not as sensitive as isotope incorporation but nonetheless adequate. Chloroquine (500ug ml^{-1}) had little observable affect on DNA synthesis, but the higher concentration of the drug (5mg.ml^{-1}) prevented the increase in DNA synthesis after 30 min, and DNA was lost from the cells after synthesis ceased (Fig. 24).

These results show a number of interesting features. Chloroquine (500ug.ml^{-1}) affected both RNA and protein synthesis significantly as soon as it was added, yet there was no visible effect on the increasing turbidity of the culture; as stated above RNA synthesis was much more sensitive to chloroquine inhibition than protein. Chloroquine (5mg.ml^{-1}) prevented synthesis of RNA and DNA before

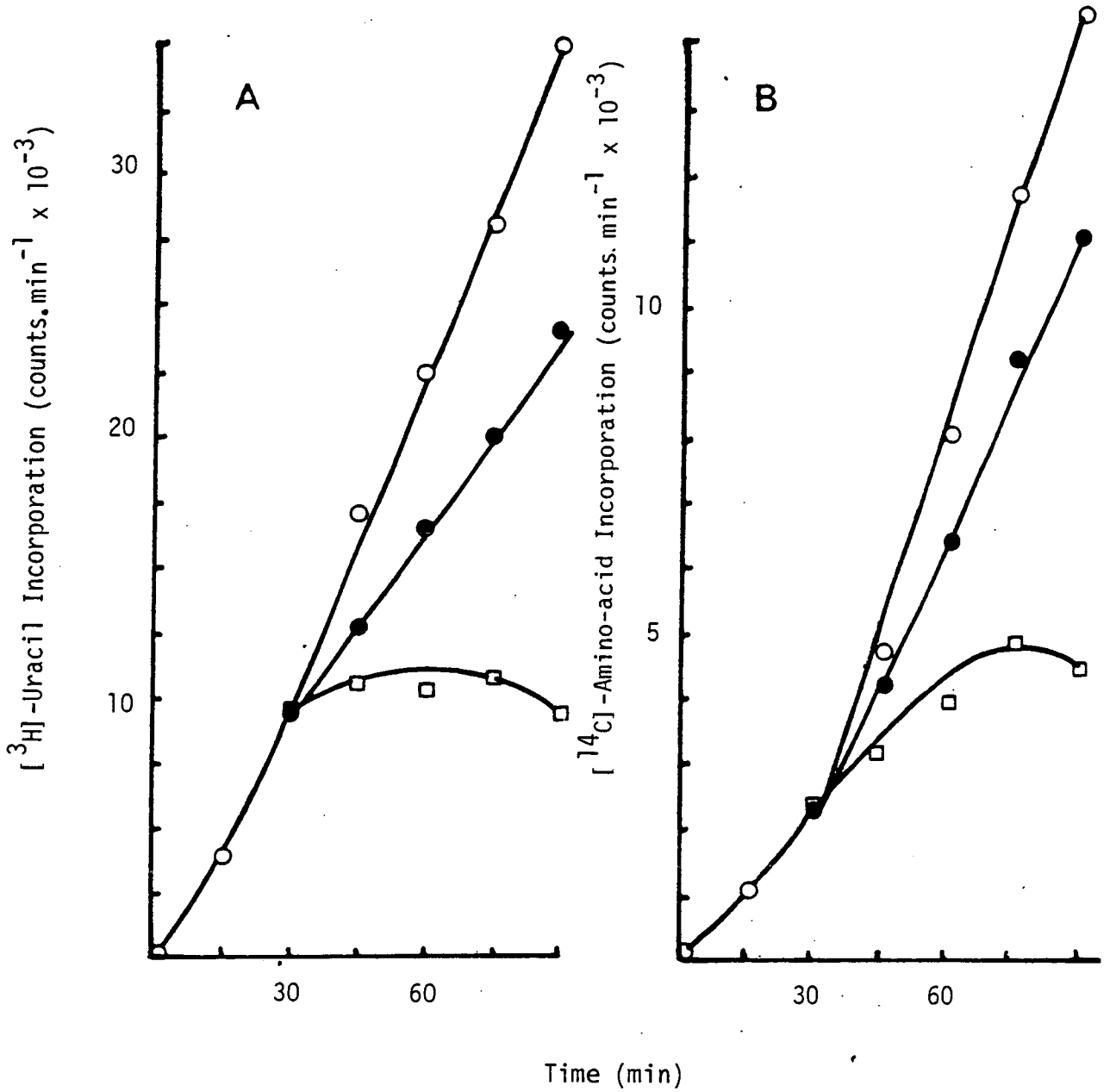


Figure 23. Effect of chloroquine on RNA and protein synthesis during vegetative growth. Cells were grown in minimal medium, supplemented as described in the methods. At 30 min 3ml of culture was removed and chloroquine (500ug ml^{-1} or 5mg ml^{-1}) added. RNA synthesis (A) and protein synthesis (B) were measured as the uptake of [^{3}H]-uracil or [^{14}C]-amino-acids, respectively into TCA-insoluble material: (○), no chloroquine; (●), 500ug ml^{-1} chloroquine; (□), 5mg ml^{-1} chloroquine

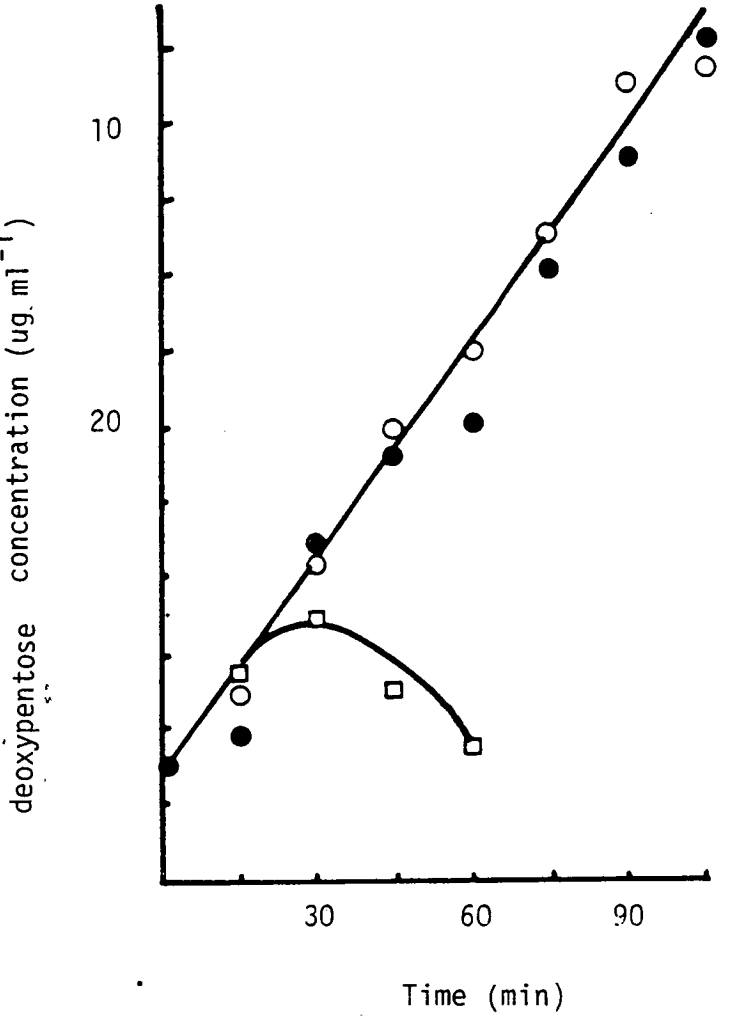


Figure 24. Effect of chloroquine on DNA synthesis in vegetative cells. Vegetative cells were grown in minimal medium, duplicate 10ml samples of culture were removed and the DNA extracted and measured following the method of Burton (1956) using 2-deoxyadenosine as standard: (○), no chloroquine; (●), chloroquine (500ug ml^{-1}) added at zero time; (□), chloroquine (.5mg ml^{-1}) added at zero time.

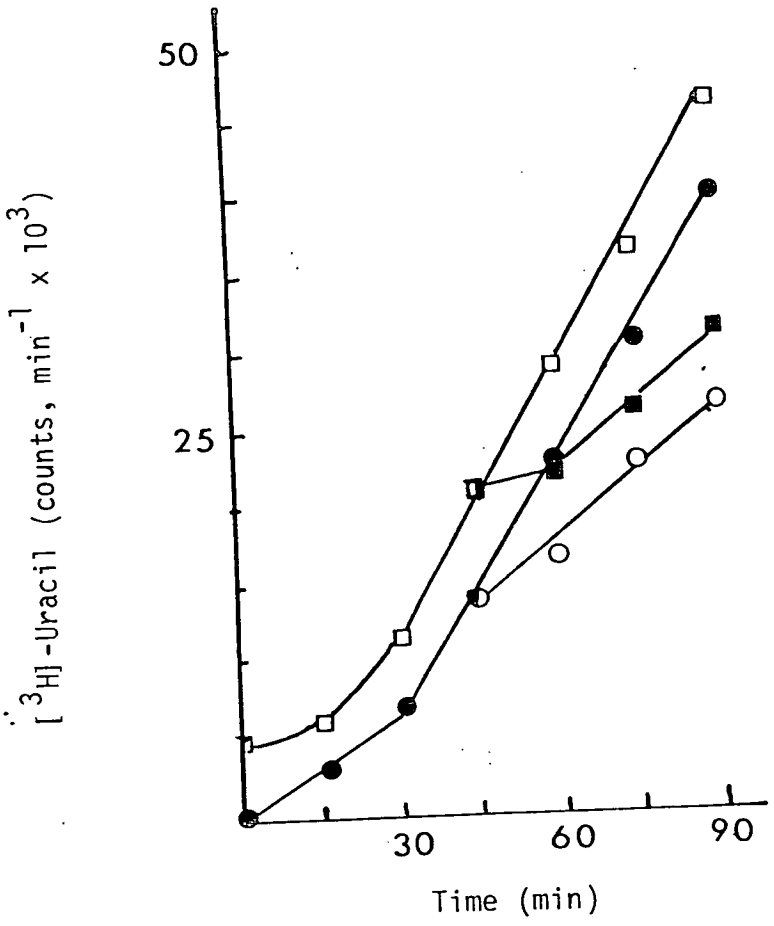


Figure 24A. Effect of chloroquine on uptake and incorporation of [^{3}H]-Uracil in vegetative cells. Vegetative cells were grown in minimal medium with the additions described previously. Samples (0.5ml) were taken and assayed for uptake and incorporation of label into RNA as described in the methods. No chloroquine, uptake (□) and incorporation (●); chloroquine (500 ug ml^{-1}) added at 45 min; uptake (■) and incorporation (○).

protein synthesis, again the effect on RNA was immediate - for DNA there was a delay of about 30 min. The breakdown of all three macromolecules was consistent with the lysis of cells discussed in chapter 3. Overall, it is probable that chloroquine preferentially inhibits RNA synthesis and so affects protein synthesis indirectly. Since the rate of protein synthesis observed was not greatly inhibited by chloroquine ($500ug.ml^{-1}$) whereas the rate of RNA synthesis was inhibited by about 30% it is possible that chloroquine prevented synthesis of stable RNA (rRNA or tRNA) preferentially, with almost normal levels of mRNA synthesised to account for the level of protein synthesis observed. Another possibility is that chloroquine can prevent uptake of [^{3}H]-uracil rather than RNA synthesis per se this possibility is examined below.

Total [^{3}H] -uracil in cells was measured in the presence and absence of $500ug.ml^{-1}$ chloroquine, 32% less label was taken up when chloroquine was present, again incorporation into TCA-insoluble material was decreased by 35% (See facing page). While this would account for the apparent drop in RNA synthesis observed another factor may be involved. In bacteria, uracil is incorporated into RNA via a "salvage" mechanism in which the uracil is converted to uridylic acid by a reaction with 5-phosphoribosyl-1-pyrophosphate before its incorporation into RNA (Mahler & Cordes, 1968). Such pathways make a minor contribution to overall RNA synthesis and it is possible that chloroquine either by inhibiting the "salvage" pathway or RNA synthesis will cause a feedback inhibition of uracil uptake. Two experiments can be done to clarify the observed inhibition of [^{3}H]-uracil uptake. Either one can use an uracil auxotroph in the uptake/incorporation experiments, in this case all RNA will be synthesised from exogenous uracil and so be measured

directly or RNA synthesis, can be measured directly by a chemical method.

RNA synthesis measured by the orcinol method described by Herbert et al.(1971) is shown in Figure 25. Chloroquine (500ug ml^{-1}) inhibited the rate of RNA synthesis by 33% from the time it was added. Therefore it is likely that the reduced uptake of uracil is due to feedback inhibition of uracil uptake, however chloroquine did inhibit RNA synthesis and the inhibition observed in the study of incorporation of [^{3}H]-uracil into RNA was real.

If as suggested earlier, chloroquine acts by preventing synthesis of stable RNA it is possible that the preferential inhibition of spore outgrowth reflects a greater requirement for synthesis of stable RNA species for outgrowth and differential transcription from these genes. The specificity of DNA-dependent RNA polymerase changes during outgrowth (see chapter 1, section 4.0) and it is possible that the polymerase itself is more susceptible to chloroquine during early outgrowth or the drug binds to genes (or their promoters) that are essential to outgrowth and so prevent transcription.

Effect of chloroquine on DNA-dependent RNA polymerase activity

In order to test the susceptibility of the RNA polymerase of outgrowing spores and vegetative cells to chloroquine a semi-in vitro system was used in which cells were permeabilized to the necessary substrates by cold-shock (Fisher et al., 1975). There are several advantages in using this system; for example the danger of losing regulatory subunits during enzyme purification is minimized; and, the DNA substrate is more akin to its natural form than is purified DNA.

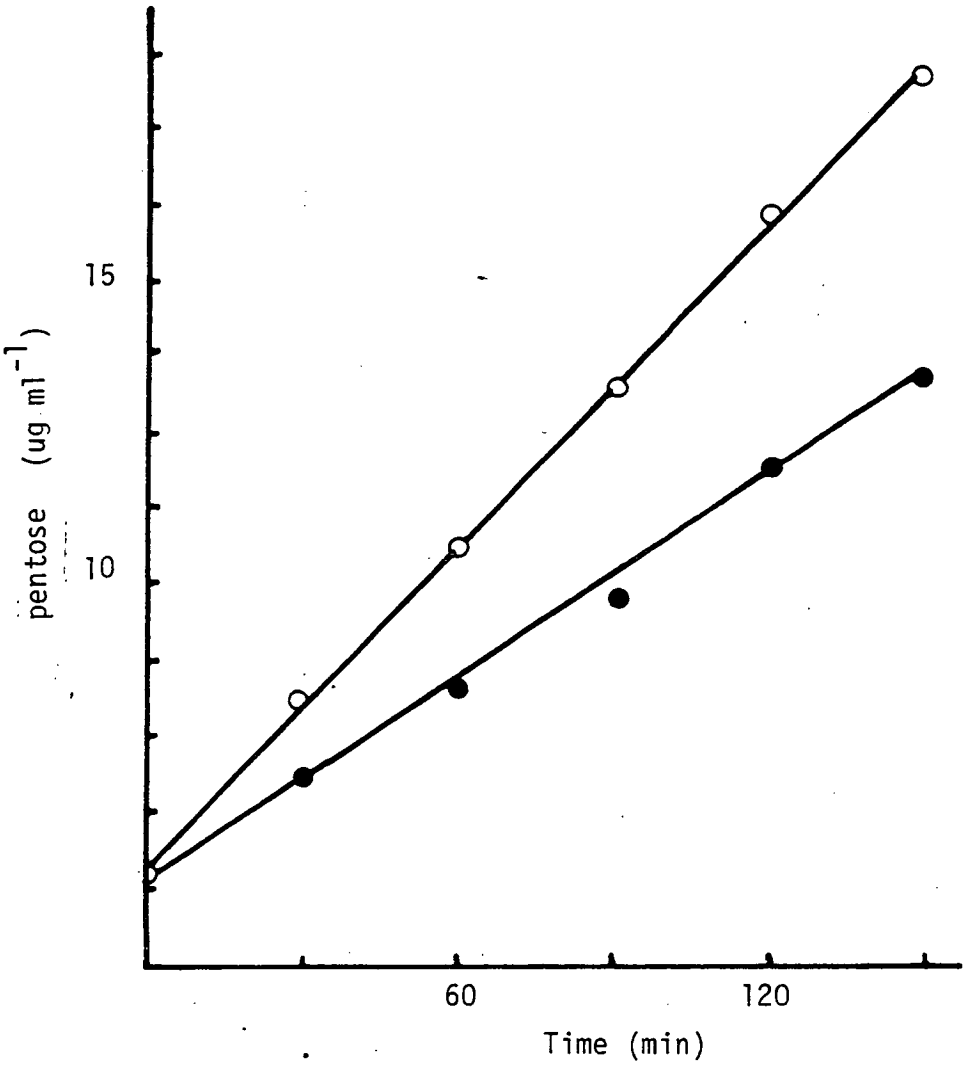


Figure 25. Effect of chloroquine on synthesis of total RNA in vegetative cells. Vegetative cells were grown in minimal medium. Duplicate 3ml samples of culture were removed and the RNA extracted and measured as described by Herbert et al.(1971) ribose was used as standard: (○), no chloroquine; (●), chloroquine (500ug ml^{-1}) added at zero time.

Figure 26 shows the time course of RNA-polymerase activity as measured by incorporation of [^{3}H]-UTP into TCA-insoluble material in permeabilized vegetative cells. The assay was linear for at least 20 min, and addition of rifampicin (a specific RNA-polymerase inhibitor) reduced activity to about 6% of that seen in untreated cells, therefore the activity of the RNA-polymerase was being measured. As rifampicin inhibits the initiation step of transcription, the residual activity seen in the presence of rifampicin was probably due to the elongation of previously initiated chains and Fisher et al.(1975) have shown that the rifampicin-resistant synthesis of RNA was equivalent to that occuring in the first minutes of incubation in an untreated control.

Addition of chloroquine to the assay mixture led to inhibition of this RNA-polymerase activity (Fig. 27). The drug at a concentration of 500ug.ml^{-1} inhibited UTP incorporation by about 50% in early and late outgrowing spores in cells of ts-134after 1h at 45^{o}C, and in vegetative cells-; at the higher concentration of chloroquine (5mg ml^{-1}) the inhibition was not altered to much below 50%. Therefore it is unlikely that the RNA-polymerase of early outgrowing spores or ts-134 in a chloroquine sensitive state, has any greater sensitivity to the drug than the more resistant vegetative and late outgrowing cells. However, in the assay mixture Mg Cl_2 and KCl are present at concentrations of 10mM and 100mM respectively. It was shown previously (Chapter 4) that Mg Cl_2 concentrations of 0.25mM or KCl concentrations in excess of 100mM can reverse chloroquine inhibition, and thus one might expect that the relatively high concentrations of Mg^{2+} and K^+ ions in the assay mixture would interfere with the action of the drug on RNA-polymerase activity. To test this hypothesis the assay was repeated with $MgCl_2$ and

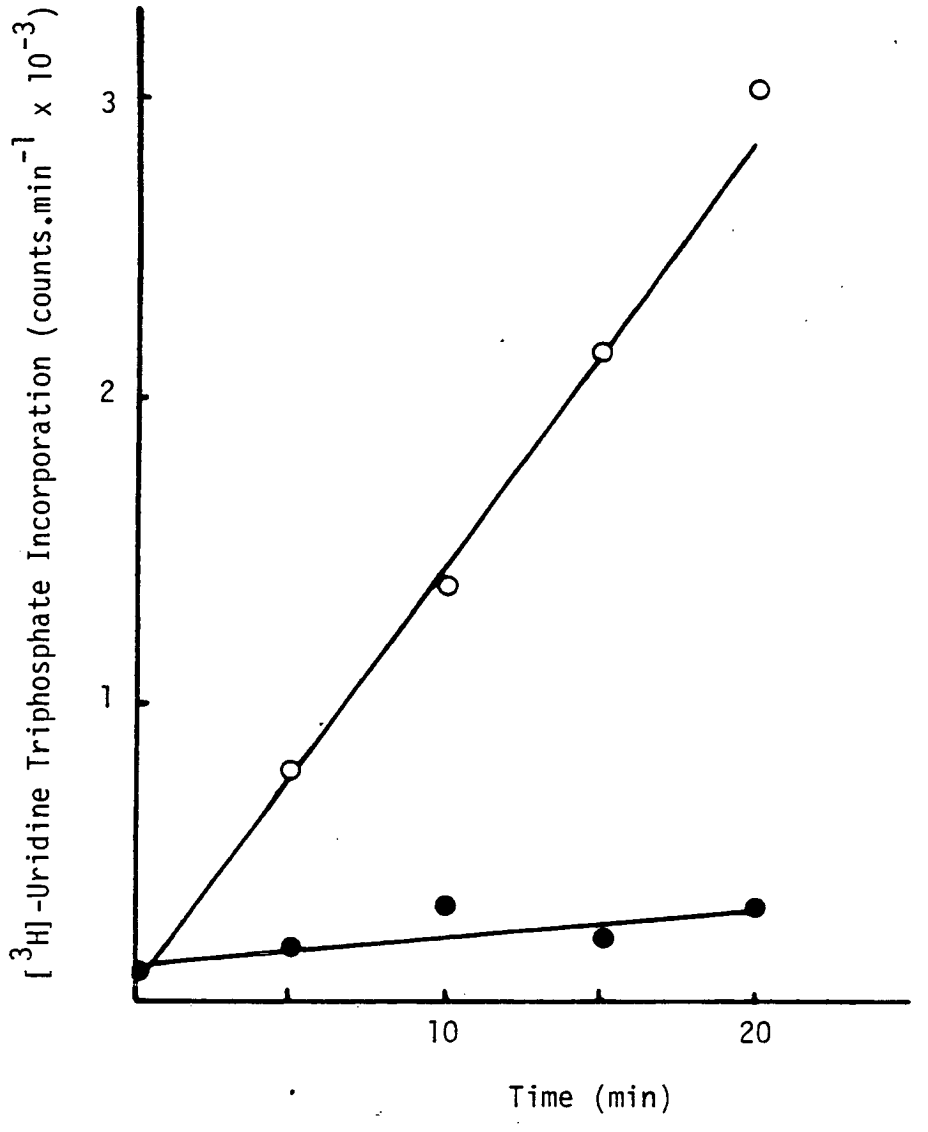


Figure 26. Time course of DNA-dependent RNA-polymerase activity in a semi-in vitro assay system. Vegetative cells were treated as described in the methods and the incorporation of [^{3}H]-UTP into TCA-insoluble material was followed with time: (○), activity of control assay; (●) activity in the presence of rifampicin (2ug.ml^{-1})

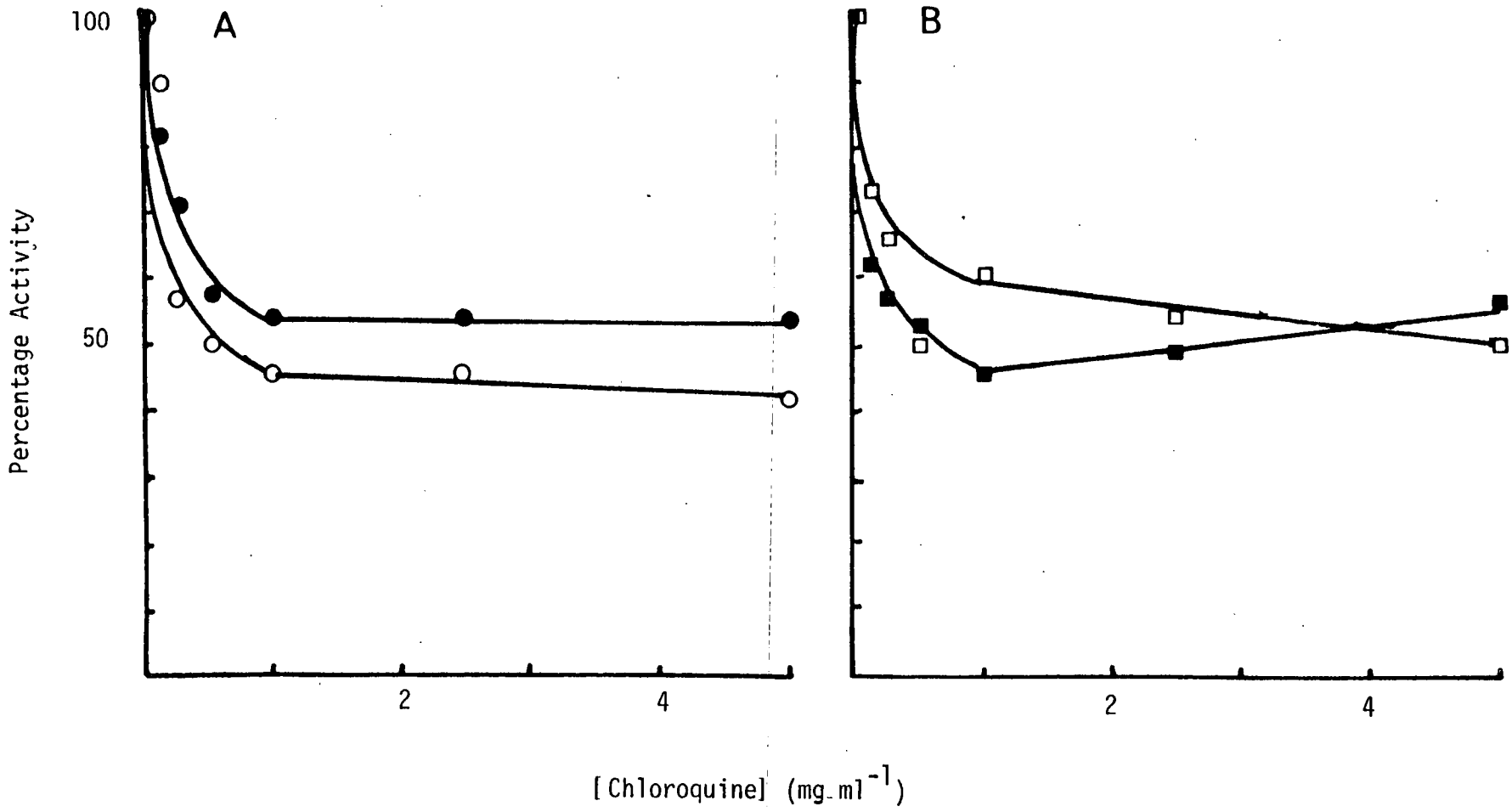

A
B
100
50
Percentage Activity
2
4
2
4
[Chloroquine] (mg.ml^{-1})

Figure 27. Effect of chloroquine on DNA-dependent RNA polymerase activity. Vegetative cells (A) and outgrowing spores (B) were treated as described in the methods. After 15 min incubation at 37°C, the incorporation of [^{3}H]-UTP into TCA-insoluble material was measured. A: (○), wild type vegetative cells; (●), ts-134 cells after 1h at 45°C. B: (□), early outgrowing spores; (■), late outgrowing spores.

KCl at 0.1mM and 50mM respectively, and although the incorporation by untreated cells was reduced by 90%, chloroquine (500ug ml^{-1}) inhibited the remaining UTP incorporation by 42%. Raising the $MgCl_2$ concentration to 1.0mM did not alter this level of inhibition (data not shown). Therefore it may be that the relief of chloroquine inhibition by $MgCl_2$ is not related to the inhibition of RNA polymerase, but it is possible that the concentrations of Mg^{++} or chloroquine in intact cells bear no relationship to those in the permeabilized cells.

Chloroquine inhibited RNA-polymerase activity to an apparent maximum of 50%, yet in outgrowing spores there was little RNA synthesis when chloroquine was present. If one assumes that this represents the state *in vivo* then it is possible that chloroquine is binding preferentially to genes specific to or essential for outgrowth, but not for vegetative growth, and if transcription from such genes were prevented, further spore development would cease. There is evidence that during early outgrowth, genes are read preferentially from DNA rich in G/C bases (Setoguchi *et al.*, 1978). Also gramicidin S, a preferential inhibitor of spore outgrowth in *Bacillus brevis* has been shown to bind preferentially to G/C rich DNA (Lazaridis *et al.*, 1980; Sarkar *et al.*, 1978). The possibility that chloroquine inhibits transcription of only certain classes of genes was examined by comparing its effect with actinomycin D, a drug that binds specifically to G/C base-pairs in DNA (Goldberg & Friedman, 1971). Table 12 shows the results obtained when mixtures of chloroquine and actinomycin D were added to the semi-*in vitro* RNA-polymerase system, the inhibitory effects of both drugs were additive and there was no indication that there was any interaction between the drugs. If chloroquine were binding preferentially to G/C base pairs in DNA one might have expected inhibition of

Table 12

Inhibition of RNA polymerase activity by mixtures of chloroquine and actinomycin D

Inhibitor ($ug.ml^{-1}$)		Observed inhibition (%)	Expected inhibition
Actinomycin D			
2		21	-
4		32	-
6		40	-
Chloroquine			
100		19	-
500		45	-
Actinomycin D	Chloroquine		
2	100	40	40
2	500	70	66
4	100	55	51
4	500	72	77
6	100	49	59
6	500	73	85

Vegetative cells were permeabilized and incorporation of [^{3}H]-UTP into TCA-insoluble material was measured after 15 min as described in the methods. The observed inhibition is the percentage decreased in UTP incorporation observed after adding the inhibitor. The expected inhibition is the sum of the observed inhibition in the presence of chloroquine and the observed inhibition in the presence of actinomycin D.

the polymerase to have reached a plateau since the maximum level of inhibition of this system seen in the presence of actinomycin D (8-10ug $.ml^{-1}$) was 65% and in the presence of chloroquine about 50%. However, mixtures of both drugs inhibit the incorporation of UTP above both these levels. The near perfect additivity of inhibition may mean that the drugs are inhibiting different things e.g. actinomycin D binding to G/C rich regions and chloroquine binding to A/T rich regions of DNA or preventing initiation of transcription of the polymerase.

Conclusions

The effect of chloroquine on RNA, protein and DNA synthesis has been examined in outgrowing spores and vegetative cells.

Chloroquine prevented large scale RNA, protein and DNA synthesis when added to germinating spore suspensions. The same concentration of chloroquine (500ug ml^{-1}) added to outgrowing spores after germ-cell emergence inhibited the rate of RNA, protein and DNA by 30%, 50% and 20% respectively. Outgrowing spores escaped from chloroquine inhibition concurrent with the onset of large scale synthesis of the major macromolecules examined. These results show good correlation with the observations made in Chapter 4, that chloroquine prevented emergence of the germ-cell from the spore coat and that outgrowing spores became resistant to chloroquine inhibition on emergence of the germ-cell. It is probable that during early outgrowth chloroquine prevents RNA synthesis since although there was little protein and DNA synthesis in the presence of chloroquine, synthesis of both macromolecules during outgrowth requires prior transcription (Kobayashi et al., 1965).

Chloroquine inhibition was independent of DNA synthesis during outgrowth but it is possible that chloroquine acts by preventing the repair-like DNA synthesis that occurs during early outgrowth (Chapter 4). However, this "repair" was resistant to chloroquine and therefore it is unlikely that it has any bearing on chloroquine inhibition.

Macromolecular synthesis during vegetative growth was relatively resistant to the action of lower concentrations of chloroquine (500ug ml^{-1}) but further synthesis of RNA, protein and DNA was prevented after a lag

period in the presence of 5mg ml^{-1} chloroquine, and this was followed by breakdown of the macromolecules. Nevertheless chloroquine (500ug ml^{-1}) had an effect on RNA and protein synthesis during vegetative growth, the rate of RNA synthesis was reduced by 30% while the rate of protein synthesis was only reduced by 9%. DNA synthesis was apparently unaffected by chloroquine and although it was measured by the diphenylamine method (which is less sensitive than isotopic labelling) any large scale inhibition would show up. In previous reports of chloroquine action in Plasmodium knowlesi and B. megaterium, the authors have suggested that DNA synthesis is the main target (Polet & Barr, 1968; Ciak & Hahn, 1966), this does not appear to be the case here.

Synthesis of various RNA classes

If transcription is affected, it could be that chloroquine acts on synthesis of any or all of thethree classes of RNA: mRNA, rRNA or tRNA. From the experiment with vegetative cells, using the inhibitory concentration of chloroquine (5mg ml^{-1}), it may be concluded that rRNA or tRNA synthesis was more susceptible to inhibition since protein synthesis continued for some time after addition of the drug. Rodenberg et al. (1968) have shown that the rate of protein synthesis is proportional to the mRNA population and not the amount of total RNA or rRNA present.

Chloroquine could affect synthesis of stable RNA species (rather than mRNA) by either (i) causing a nutritional shift down or (ii) binding preferentially to rRNA or tRNA cistrons (Nierlich, 1978). As far as is known during outgrowth the normal mechanisms for induction/repression of protein synthesis apply (Spiegelman et al., 1969),

but to date there is little known about the regulation of rRNA synthesis.

Another explanation of the effect of chloroquine on transcription is that the synthesis of certain mRNA's during outgrowth is more sensitive to chloroquine inhibition. This would account for the observation that protein synthesis was apparently more sensitive to chloroquine (500ug ml^{-1}) when the drug was added to outgrowing spores after escape time. It is well known that the specificity with which DNA-dependent RNA polymerase reads from gene promoters changes during sporulation and outgrowth (Chapter 1, Section 4.0), and it is possible that the RNA-polymerase itself or the genes read by the polymerase during spore outgrowth are subject to preferential inhibition by chloroquine. However, the activity of the RNA-polymerase was not preferentially inhibited during outgrowth compared to vegetative growth. Neither was there any evidence that chloroquine was binding preferentially to G/C base pairs in DNA, rather that chloroquine inhibition of transcription was unrelated to the inhibition shown by actinomycin D i.e. binding specifically to G/C pairs.

One interesting observation that may have some bearing on the increased sensitivity of outgrowth to chloroquine. For the same cell (spore) concentration the activity of RNA-polymerase was (as a percentage of that seen in vegetative cells) much less in early outgrowth spores (28%), later outgrowth spores (34%) and ts-134cells sensitized to chloroquine by exposure to 45°C for 1h (36%). A similar phenomenon was observed by Fisher et al.(1975) in sporulating cells, these workers have shown that the low activity observed was not caused by a difference in permeability of cells but rather reflected a lower rate of transcription

in the sporulating cells. Presumably this is the casein outgrowing spores and the ts mutant after it has been subjected to the restrictive temperature. It is possible that if chloroquine is present when the rate of transcription is low, its inhibitory effect on transcription may be sufficient to prevent further growth. Certainly the rateof transcription in ts-134is reduced at 45°C (Mendelson & Gross, 1967) and is particularly low during early outgrowth of spores. It is interesting to speculate that the preparation for starting the burst of activity that begins a return to vegetative growth requires the build up of specific protein(s) to a certain level; so the course of outgrowth may be much more sensitive to transcription inhibition. The same argument can be applied to ts-134which requires a lag-phase after treatment at 45°C before normal growth recommences (see Chapter 4). If this were the case outgrowth would be more sensitive to inhibitors of transcription, and indeed this appears to be true e.g. acriflavin and rifampicin preferentially inhibited spore outgrowth (see Chapter 3).

The maximum level of inhibition by chloroquine of 50% RNA polymerase activity is curious; this phenomenon has been observed previously by Cohen and Yielding (1965a). In vitro much higher inhibition of transcription occurs, and the reasons for this discrepancy are not obvious although several explanations are possible: (i) the in vivo effect is not due to chloroquine acting directly on RNA-polmerase e.g. it may be due to lack of energy. If this were the case it is unlikely that protein synthesis would be able to continue in the presence of chloroquine (5mg. ml^{-1}) in vegetative cells, since protein synthesis requires energy in the form of ATP and GTP. (ii) Some specificity of transcription may be lost following permeabilization,

i.e. loss of ions etc., therefore the *in vitro* process may not have the same strict control of initiation, elongation or termination as intact cells. Furthermore, permeabilization may alter the supercoiling of DNA which would affect both transcription and the degree of binding of intercalating molecules (Gale *et al.*, 1972), such that control of transcription is lost or less chloroquine bound to DNA. (iii) Chloroquine may be acting on the RNA-polymerase rather than DNA, while there is no direct evidence for this possibility Whichard *et al.*(1972) reported that inhibition of DNA polymerase activity by chloroquine was dependent on the amount of enzyme present. More indirect evidence for this was presented earlier; the synergystic action of chloroquine and ethidium bromide (see Chapter 4), and the additive effect of chloroquine and actinomycin D may indicate that chloroquine is not acting on the same target as these two drugs i.e. binding to DNA. One alternative is that chloroquine binds to the RNA-polymerase and the full effect of this may not be apparent in the artificial environment of the permeabilized cells.

The basis of differential sensitivity

The target of chloroquine is probably transcription but there is no clear evidence that there is any aspect of RNA synthesis that differs during outgrowth. Another possibility that needs to be considered is that the drug does not get into cells to the same extent, and the difference in sensitivity of transcription in the *in vitro* system and vegetative cells to 500ug.ml^{-1} chloroquine may be due to differential uptake of the drug. This hypothesis is examined in the next chapter.

CHAPTER 6

Factors Affecting the Uptake of Chloroquine

Introduction

If the basis of preferential inhibition of spore outgrowth by chloroquine is due to its differential uptake the factors that are involved in the uptake of drugs by bacteria should be considered. The factors that affect the movement of antibiotics across the permeability barriers of bacterial cells have been outlined by Franklin (1973). Those factors pertinent to the entry of a molecule such as chloroquine into a Gram-positive organism are presented below.

Four main factors have to be considered when studying the entry of drugs into cells; the nature of the antibiotic itself; and mode of drug uptake and the permeability barriers to be crossed; and, the nature of the interaction of the drug and its target. The first two factors are closely related, the structure of the antibiotic governs the way it enters the cell and also how the permeability barriers are breached. In the case of a drug like chloroquine two features of its structure will influence its uptake. Firstly, size; water-soluble molecules of molecular mass greater than 100 cannot diffuse through a cell-membrane and chloroquine (mol mass 519) is therefore too large to pass through the cell membrane. Secondly there is charge; again large ionized molecules like chloroquine (pKa 8.1, 10.1) generally cannot diffuse across an intact membrane. However, several exceptions to this rule have been found e.g. streptomycin, penicillins, rifamycins and several other antibiotics. It is thought that although these molecules can only diffuse slowly into cells, the fact that they bind strongly to their target inside promotes a net inward flux (Franklin & Snow, 1975). Also, such molecules may distribute unequally across a membrane because of either a pH difference across the membrane or due to a Donnan

equilibrium. Other antibiotics can enter cells by facilitated diffusion or if the transfer is linked to an input of energy by active transport. These transfer systems often show specificity and in most cases the antibiotics have a similar structure to that of a cellular nutrient and compete with the natural permeant for transfer sites e.g. D-cycloserine is a structural analogue of D-alanine.

The cytoplasmic membrane is the most important barrier to many water-soluble drugs. Often on lowering the degree of ionization of a drug by changing the pH towards its pKa value its effectiveness is enhanced by increasing the lipophilic properties of the molecule. In gram-positive organisms the outer cell layers are probably not important in drug penetration, however, teichoic acids are highly charged and should influence the uptake of charged drugs. In the case of streptomycin, a positively charged molecule, the negative teichoic acids cause a build up of the drug which may increase its ability to breach the cytoplasmic membrane. Negatively-charged molecules e.g. the penicillins are apparently not repulsed by the charge on teichoic acids, because of the high sensitivity of many gram-positive organisms to the drug (Franklin, 1973).

In this chapter several of the factors which may effect the uptake of chloroquine have been examined and the results related to the preferential inhibition of spore outgrowth by the drug.

Effect of hydrogen ion concentration on chloroquine inhibition

In chapter five it was suggested that because chloroquine inhibited transcription more in cells with no permeability barrier than in growing cells, the difference may have been due to uptake of the drug, therefore the factors which may effect uptake were examined.

The chloroquine molecule with ionization constants of pKa_1, 10.2 and pKa_2 8.1 is present in both monovalent and divalent-anion forms at pH 7.0 (Irvin & Irvin, 1947, Fig. 1) by altering the pH of the medium it is possible to vary the ratio of monovalent to divalent ions. If one ionic species of chloroquine is taken up selectively it is possible to identify the preferred form from the change in minimum inhibitory concentration (mic) with pH. The results of experiments done using pH values from 6 to 8 are shown in Table 13. There is a clear relationship between the amount of the monovalent (and therefore more lipophilic) form of the drug present and the mic. Therefore, the monovalent form predominates in penetrating the cell. It is of particular interest that at pH 8 the mic for outgrowth and vegetative cells is the same. Microscopic examination of both outgrowing spores and vegetative cells in the presence of 100ug ml^{-1} chloroquine at pH 8 showed the same features of inhibition discussed in Chapter 4, i.e. germinated spores continued to swell up to germ-cell emergence and vegetative cells lysed. This was confirmed turbidometrically, pre-germinated spores showed almost identical changes in turbidity in the presence of 500ug ml^{-1} chloroquine at pH 7.0 and 8.0 and 100ug ml^{-1} at pH 8.0 (Fig. 29), similarly vegetative cells continued to grow for 90 min after adding 100ug ml^{-1} chloroquine at pH 8.0, thereafter the cells lysed (Fig. 30). It is, therefore, unlikely that a different function is inhibited at pH 8.0 and furthermore, it is unlikely that chloroquine inhibits a function which differs between outgrowing spores and vegetative cells, rather that outgrowing spores are permeable to both species of chloroquine whereas vegetative cells

Table 13. Inhibitory Effect of Chloroquine at Different pH Values

pH	Mic Chloroquine [ug ml^{-1}] Outgrowth	Vegetative Growth
6	2500 (1.2)	6000
7	500 (11.1)	5000
8	100 (55.7)	100

Spores and vegetative cells were grown in NB adjusted to the required pH with HCL or NaOH. Chloroquine was added at zero time. Growth at $30^{o}C$ was followed for 150 min. The figures in parentheses represent the percentage chloroquine in the monoprotonated form.

are not.

The mechanism of differential chloroquine permeability

There are several possible explanations for the preferential entry of charged molecules into outgrowing spores : either the membrane or cell wall alters during outgrowth, or the mechanisms of drug entry change. Teichoic acids are not present in dormant spores but are synthesized during spore outgrowth (Boylen & Ensign, 1968). As mentioned previously teichoic acids are strongly polar and provide a negatively charged outer surface to the cell which may influence the penetration of ionized molecules into the cell. In order to assess the relevance of teichoic acids to chloroquine inhibition, vegetative cells were grown in a low phosphate medium medium in which teichoic acid synthesis is replaced by teichuronic acid synthesis (Tempest et al., 1968). If teichoic acids were influencing chloroquine uptake it might be expected that when cells had replaced teichoic acid with teichuronic acid in the cell-wall an altered sensitivity to chloroquine would be seen. Figure 31 shows the effect of chloroquine (500ug ml^{-1}) on vegetative growth in low phosphate medium , there was no increased inhibition by chloroquine, rather growth was stimulated, possibly due to the addition of chloroquine as its diphosphate salt.

However, in the absence of any clear evidence that the conditions were phosphate limiting, no assessment of the relevance of these results can be made.

Possible changes in membrane permeability to drugs was tested using a series of alkyl-*p*-hydroxy-benzoates. These antibiotics probably act on the cell membrane, and they have certain characteristics that are useful in studying membrane properties. First, the antimicrobial activity of alkyl-*p*-hydroxybenzoates increases with increasing chain length, i.e. the mic falls with

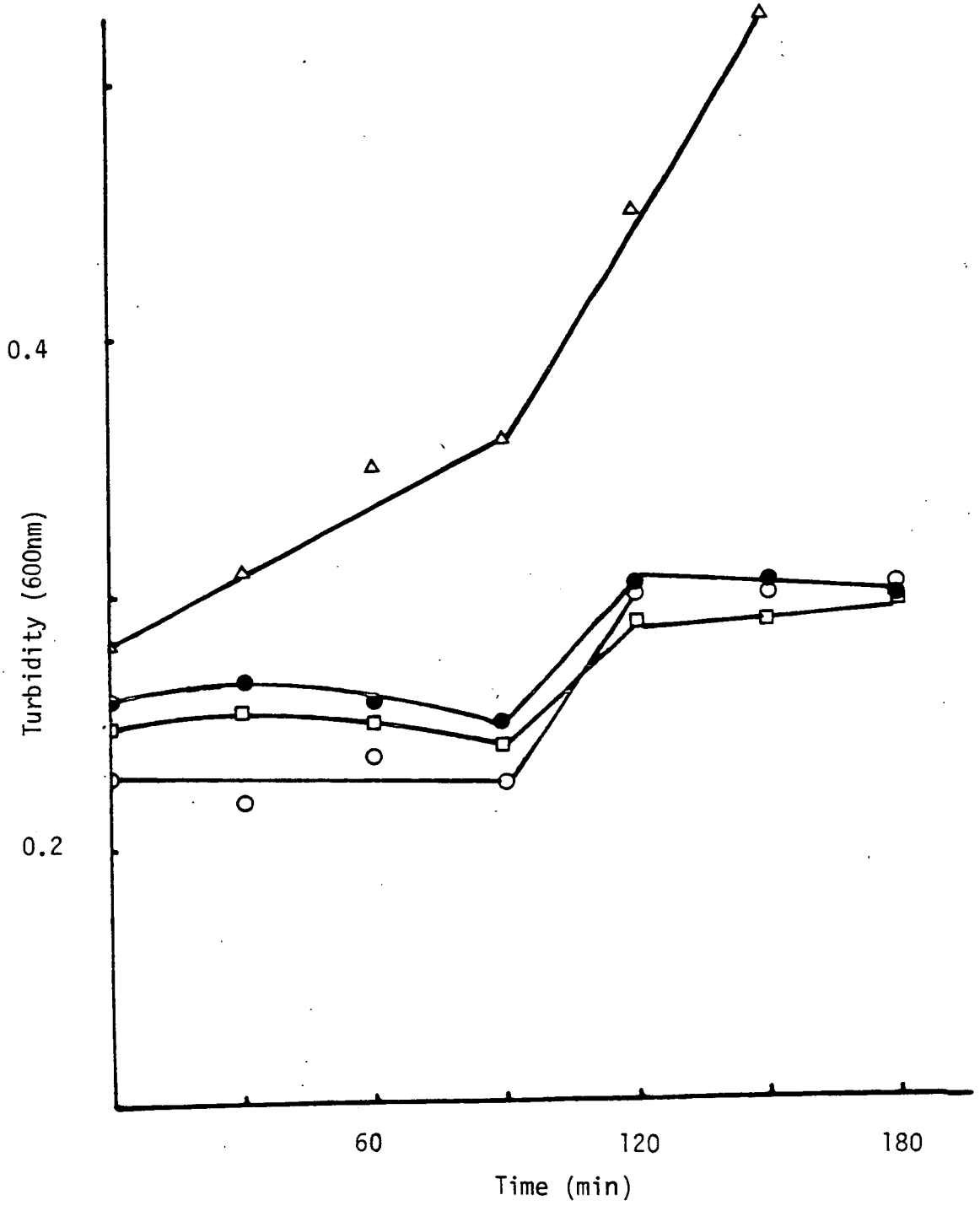


Figure 29. The effect of pH on chloroquine inhibition of outgrowing spores. Spores were pre-germinated in 100mM Na phosphate buffer, pH 7.0 containing 10mM L-alanine, centrifuged and resuspended in pre-warmed NB adjusted to the required pH with HCl or NaOH.
Chloroquine was added at zero time,
(△), pH6, chloroquine (500ug ml^{-1})
(○), pH7, chloroquine (500ug ml^{-1})
(●), pH8, chloroquine (500ug ml^{-1})
(□), pH8, chloroquine (100ug ml^{-1})

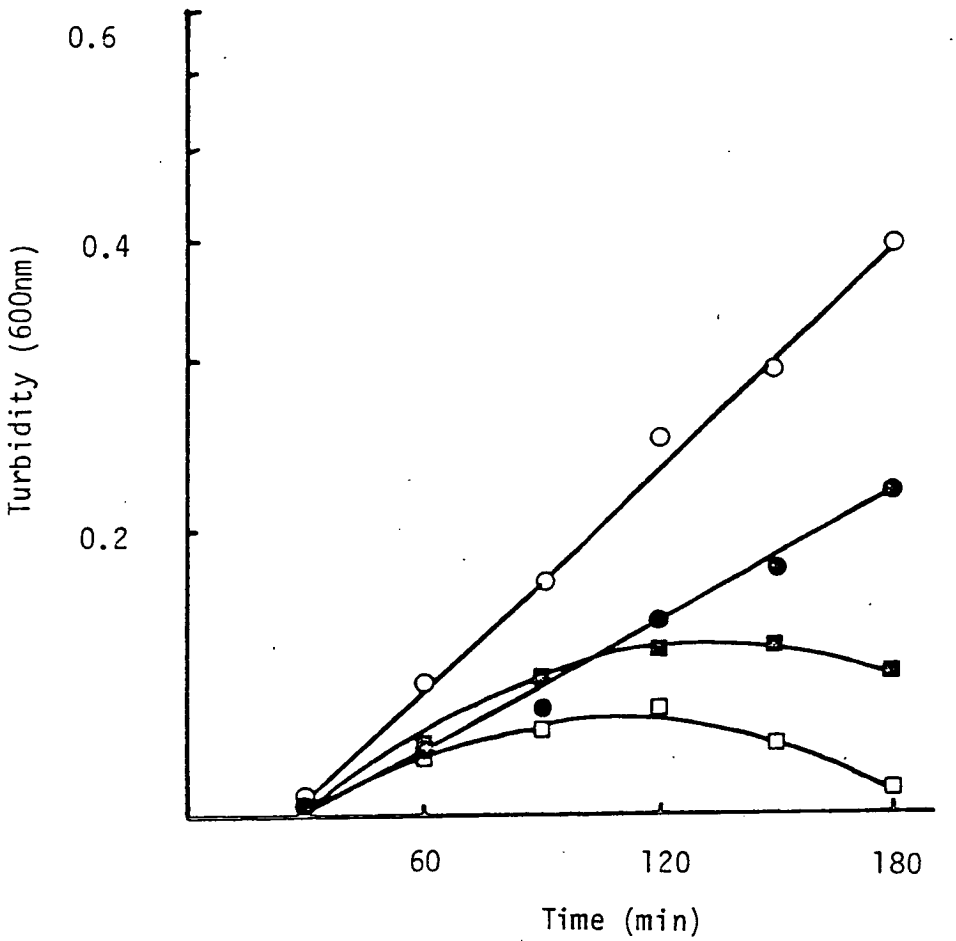


Figure 30. The effect of pH on chloroquine inhibition of vegetative cells. Cells were grown in NB adjusted to pH 8 with NaOH. Chloroquine was added at zerotime. (○), no chloroquine; (●), 50 ug ml^{-1} chloroquine; (□), 100ug ml^{-1} chloroquine; (■), 150ug ml^{-1} chloroquine.

the increasing lipid solubility of the molecule. Secondly, the mic of the drugs for bacteria varies with pH, dependent on the degree of ionization of the hydroxyl, group (Watanabe & Takesue, 1976; Dring, personal communication). Therefore these antibiotics should give sensitive probes to study the permeability of the membrane on the basis of lipid solubility and change. Table 14 shows the mic's obtained for methyl, ethyl, propyl and butyl-p- hydroxybenzoate at a range of pH values, on germinating and outgrowing spores and vegetative cells. The mic fell with the increase in the length of the alkyl chain, furthermore the logarithm of the concentration decreased linearly with this increase in the carbon number of the alkyl chain (Fig. 32). as previously shown by Watanabe and Takesue (1976). The mic fell as pH was decreased, however, there was no difference between the mic of outgrowing spores and that of vegetative cells for any of the alkyl-p-hydroxybenzoates at any pH. Therefore there is no noticeable difference in membrane sensitivity of outgrowing spores and vegetative cells to a variety of alkyl-p-hydroxybenzoates in charged or uncharged forms. One particularly interesting feature of alkyl-p-hydroxybenzoate inhibition is the increased sensitivity of germination to the molecules compared to outgrowth and vegetative growth, similar results have been reported with B. megaterium (Watanabe & Takesue, 1976), and these drugs may prove useful in germination studies.

In the absence of any involvement of teichoic acids or changes in cell-membrane in the possible increased permeability of outgrowing spores to chloroquine, the effect of a number of agents on chloroquine inhibition were examined by growing cells in the presence of sub-lethal concentrations of the test drug and a sub-lethal concentration of chloroquine (500ug ml^{-1}). Compounds which act on the cell surface, ethanol, phenethyl alcohol (2 phenyl-

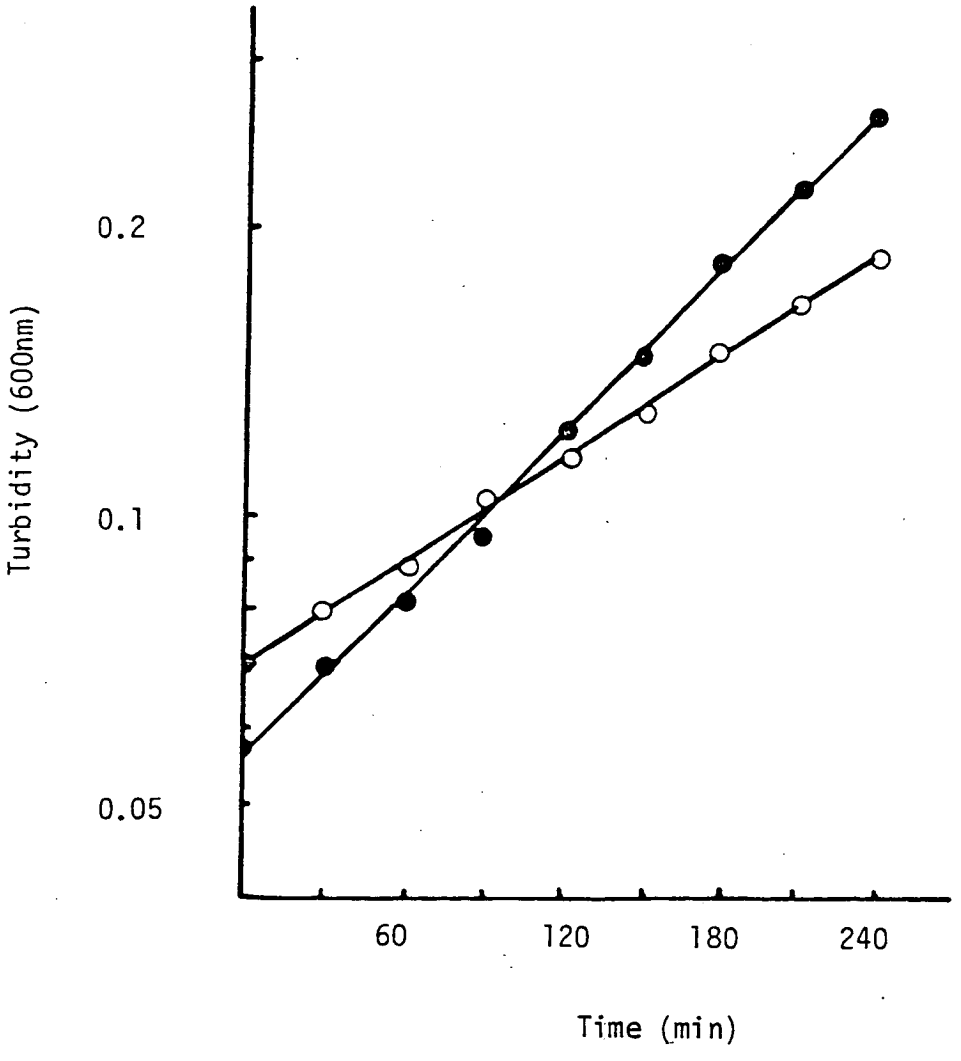


Figure 31. Effect of chloroquine on vegetative growth in low-phosphate medium. Cells were pre-grown in low phosphate medium and chloroquine (500ug ml^{-1}) added at zero time. (○), no chloroquine; (●), with chloroquine.

Table 14. Mic of alkyl-p-oH benzoates with changing pH and alkyl chain length.

pH	GERMINATION mic (mM)				OUTGROWTH/VEGETATIVE mic (mM)			
	METHYL	ETHYL	PROPYL	BUTYL	METHYL	ETHYL	PROPYL	BUTYL
5	0.12	0.07	0.023	0.0023	-	-	-	-
6	0.9	0.53	0.33	0.19	2.25	1.45	0.925	0.57
7	7.0	2.5	1.0	0.325	11.5	4.0	2.5	0.8
8	7.0	2.5	1.0	0.325	11.5	4.0	2.5	0.8

Germination at pH 5, 6 and 7 was done in citrate/Na phosphate buffer (10^{-2}M), germination at pH 8 was done in Tris/HCL buffer (10^{-2}M) both buffers contained 10mM L-alanine. Outgrowth and vegetative growth was in NB adjusted to the required pH with HCL or NaOH. The alkyl benzoates dissolved in ethanol, were added to the medium at zero time, because ethanol is itself inhibitory at higher concentrations the mic was estimated from the level of inhibition shown by the drug at non-inhibitory ethanol concentrations (Watanabe & Takesue, 1976).

ethanol) and polymyxin (Hamilton, 1971) did not increase inhibition by chloroquine (Fig. 33; A,B,C). However inhibitors affecting energy metabolism markedly enhanced the inhibition of vegetative growth by 500ug ml^{-1} chloroquine. The metabolic inhibitors act on different functions; rotenone inhibits transfer of electrons from NADH to flavoprotein (Fig. 34 A, B), oxalate inhibits malate and succinate dehydrogenases (Fig. 35 A, B), dicyclohexyl carbodiimide (DCCD) inhibits ATPase activity (Fig. 36 A) and carbonyl cyanide m-chlorophenyl hydrazone (cccp, Fig. 36B), 2, 4-dinitrophenol (DNP, Fig. 37A), and tetrachlorosalicyl anilide (TCS, Fig. 37B) act as hydrogen ion ionophores to uncouple oxidative phosphorylation (Harold, 1972). The co-operative action of rotenone and oxalate with chloroquine was almost absent in minimal medium (Figs. 34B, 35B) this reflects the difference in energy metabolism between rich and minimal medium with glucose as the main carbon source (Hanson & Cox, 1967). The results obtained with valinomycin a K^+ ionophore, were more difficult to interpret, for valinomycin to function it was found that a K^+ concentration of 230mM was required in the medium (Shioi et al., 1978), however K^+ can relieve inhibition by chloroquine (see Chapter 4). Figure 38 (A) shows the results obtained in the presence of valinomycin, 230mM K^+ and 500ug ml^{-1} chloroquine; there was no observable increase in the inhibitory effect of chloroquine, but in the presence of 2mg ml^{-1} chloroquine, valinomycin was able to overcome the reversal of chloroquine inhibition by K^+ and the cells ceased growth and lysed (Fig. 38B). Therefore, it is likely that the failure of valinomycin/K^+ to increase the inhibitory effect of chloroquine (500ug ml^{-1}) is due to the presence of K^+, but at higher chloroquine concentrations there is sufficient K^+ to reverse the inhibition.

These results indicate that the differential sensitivity of outgrowing spores relative to vegetative cells is probably due to differences

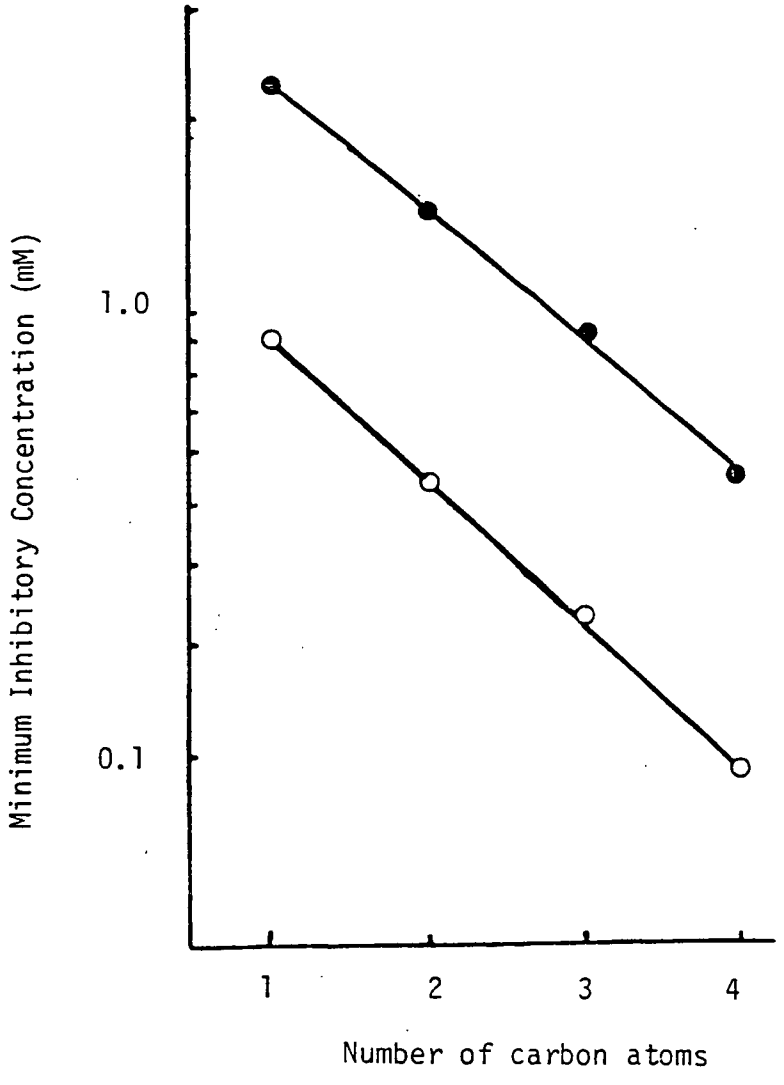


Figure 32. Relationship between the mic of spore germination and outgrowth and the number of carbon atoms in the alkyl chain of alkyl-p-hydroxybenzoate. Mic at pH6 was determined as described in Table 14. (○), germination; (●), outgrowth.

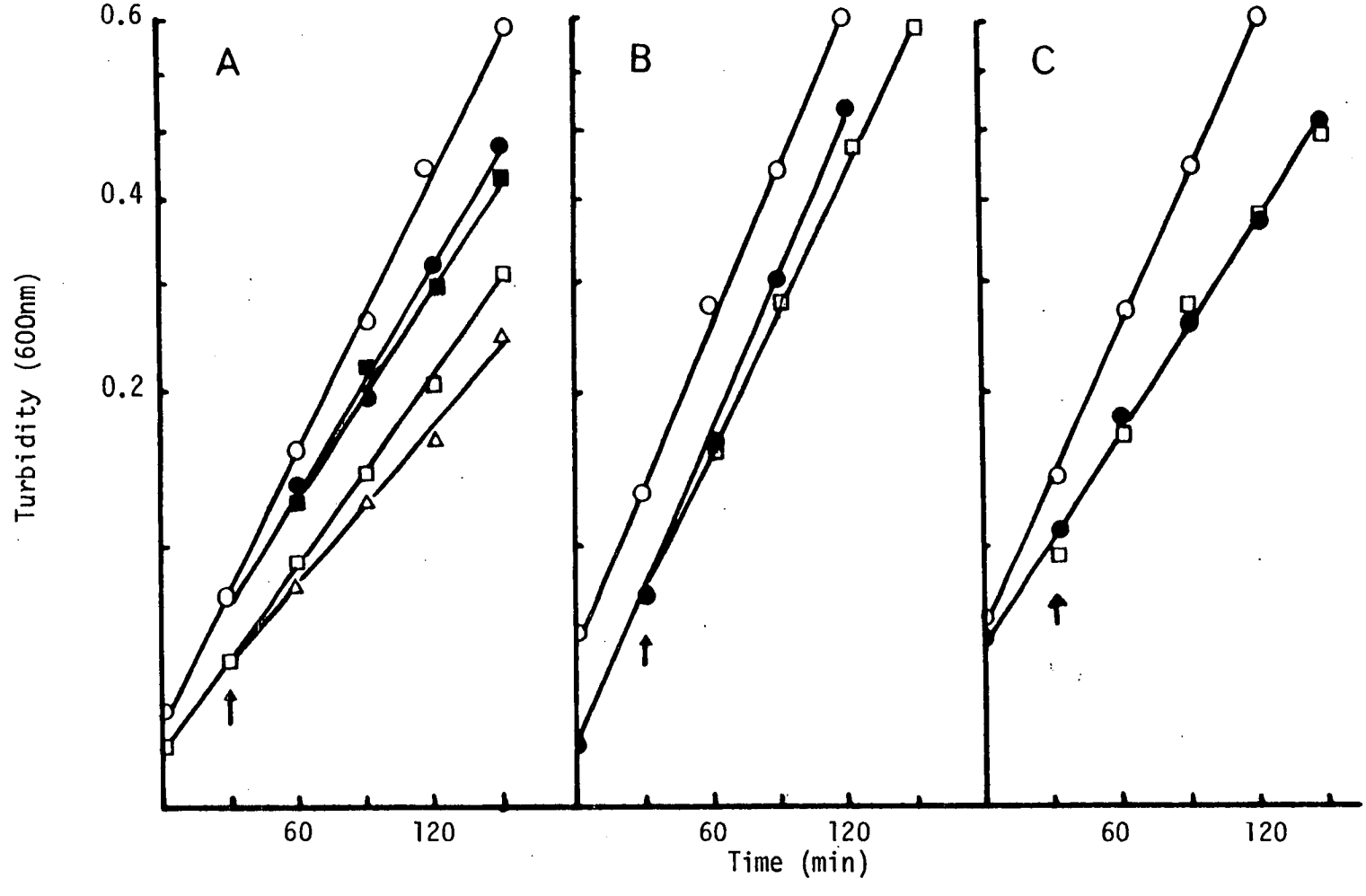


Figure 33. The effect of surface-active agents on chloroquine inhibition of vegetative growth. Cells were grown in NB. (A) Ethanol; (○), none; (●), 1% ethanol; (■), 1% ethanol and chloroquine (500ug ml^{-1}); (□), 2% ethanol; (△), 2% ethanol and chloroquine (500ug ml^{-1}). (B) Phenethyl alcohol, (○), none, (●), 1mg ml^{-1} phenethyl-alcohol (□), 1mg ml^{-1} phenethyl alcohol and chloroquine (500ug ml^{-1})
(C) Polymyxin; (○), none, (●), 5ug ml^{-1} polymyxin; (□), 5ug ml^{-1} polymyxin and chloroquine (500ug ml^{-1}). Chloroquine was added at the time indicated by the arrow.

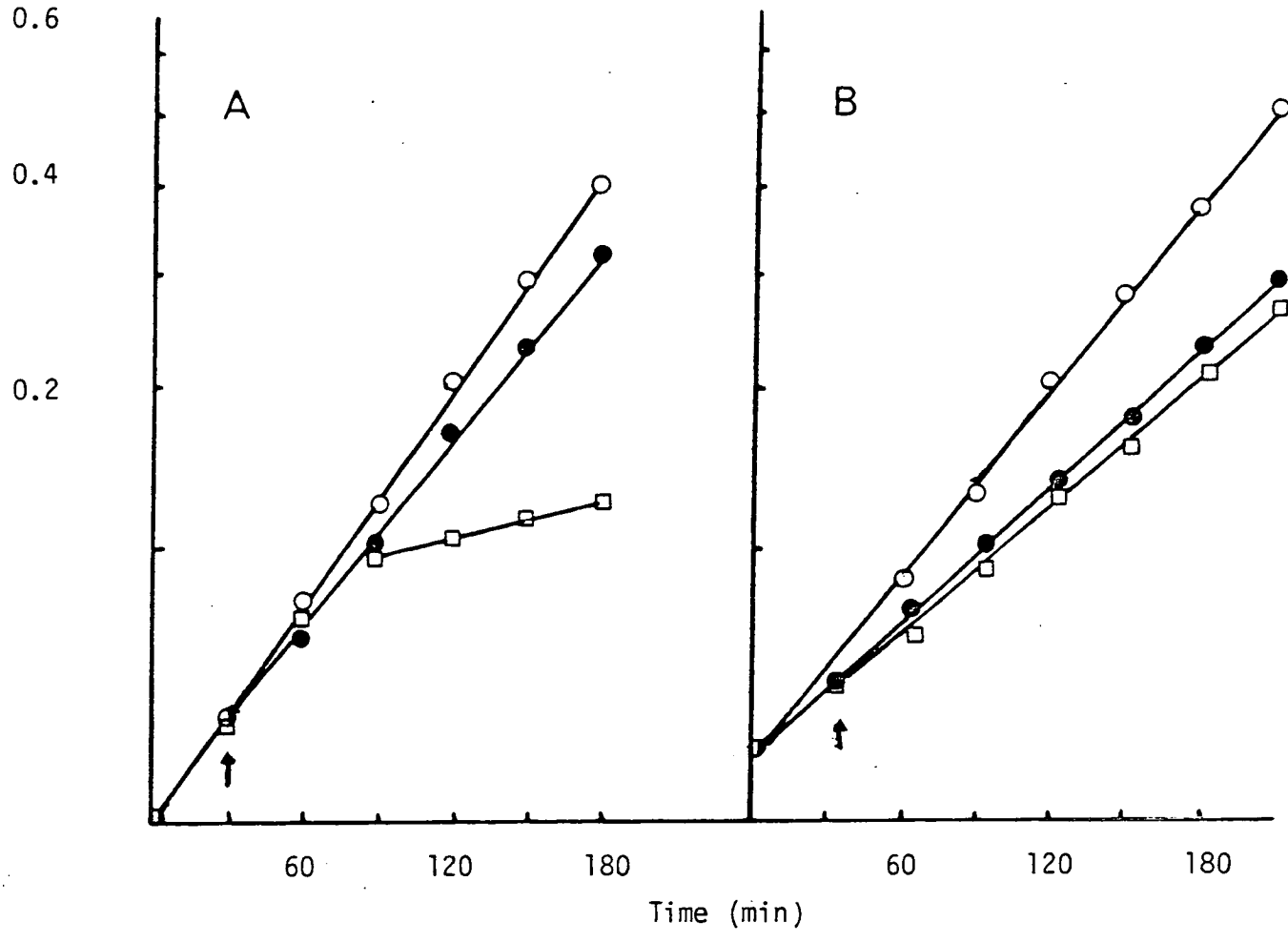


Figure 34. Effect of rotenone on chloroquine inhibition of vegetative growth. Cells were grown in NB (A) and minimal salts medium (B), (○), no addition; (●), 20ug ml^{-1} rotenone; (□), 20ug ml^{-1} rotenone and chloroquine (500ug ml^{-1}). Chloroquine was added at the time indicated by the arrow. Rotenone was present from zero time.

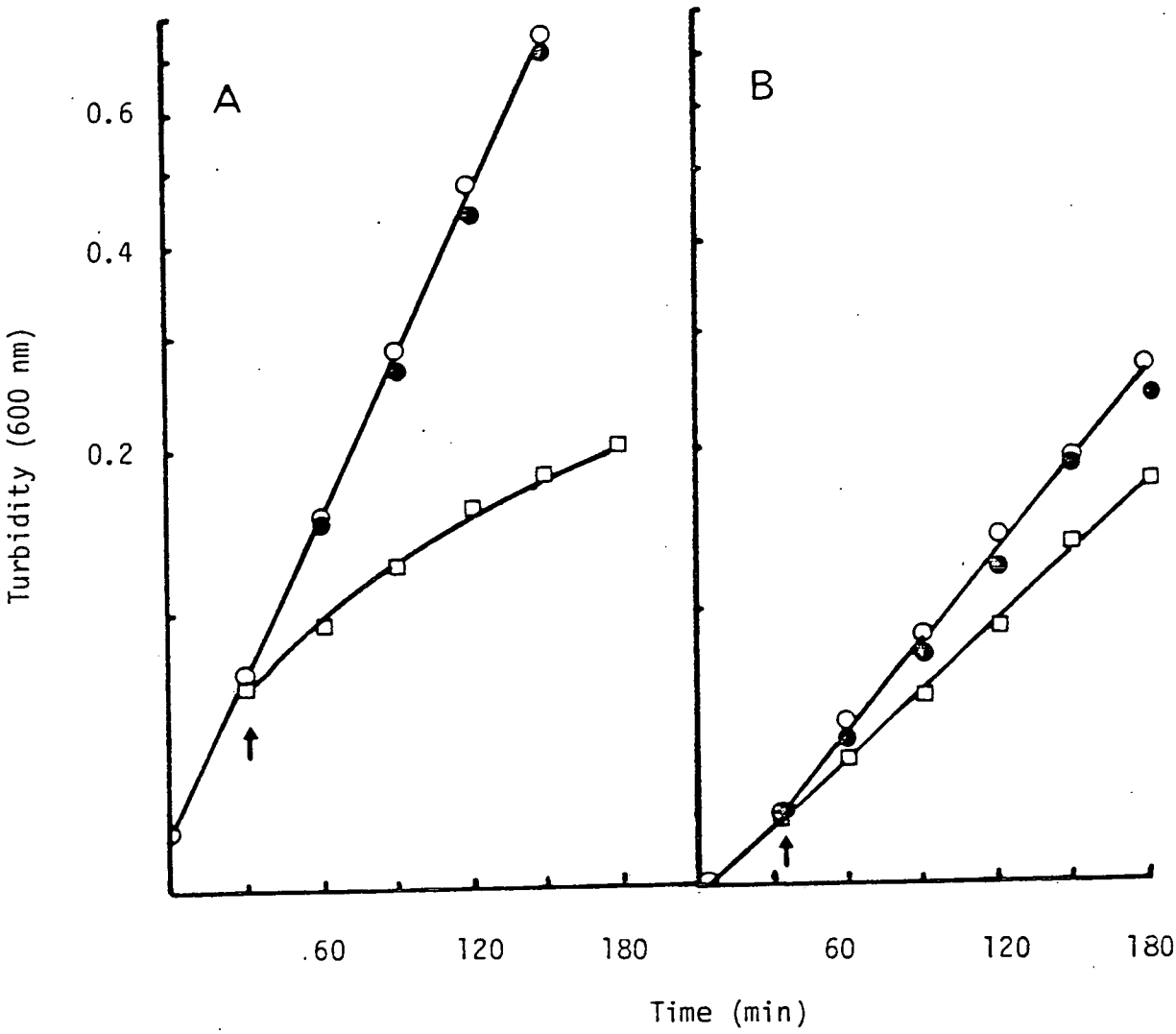


Figure 35. The effect of oxalate on chloroquine inhibition of vegetative growth. Cells were grown in NB (A) and minimal salts medium (B) (○), additions; (●), 1mg ml^{-1} NA oxalate; (□), 1mg ml^{-1} oxalate and chloroquine (500ug ml^{-1}). Chloroquine was added at the time indicated by the arrow. Oxalate was present from zero time.

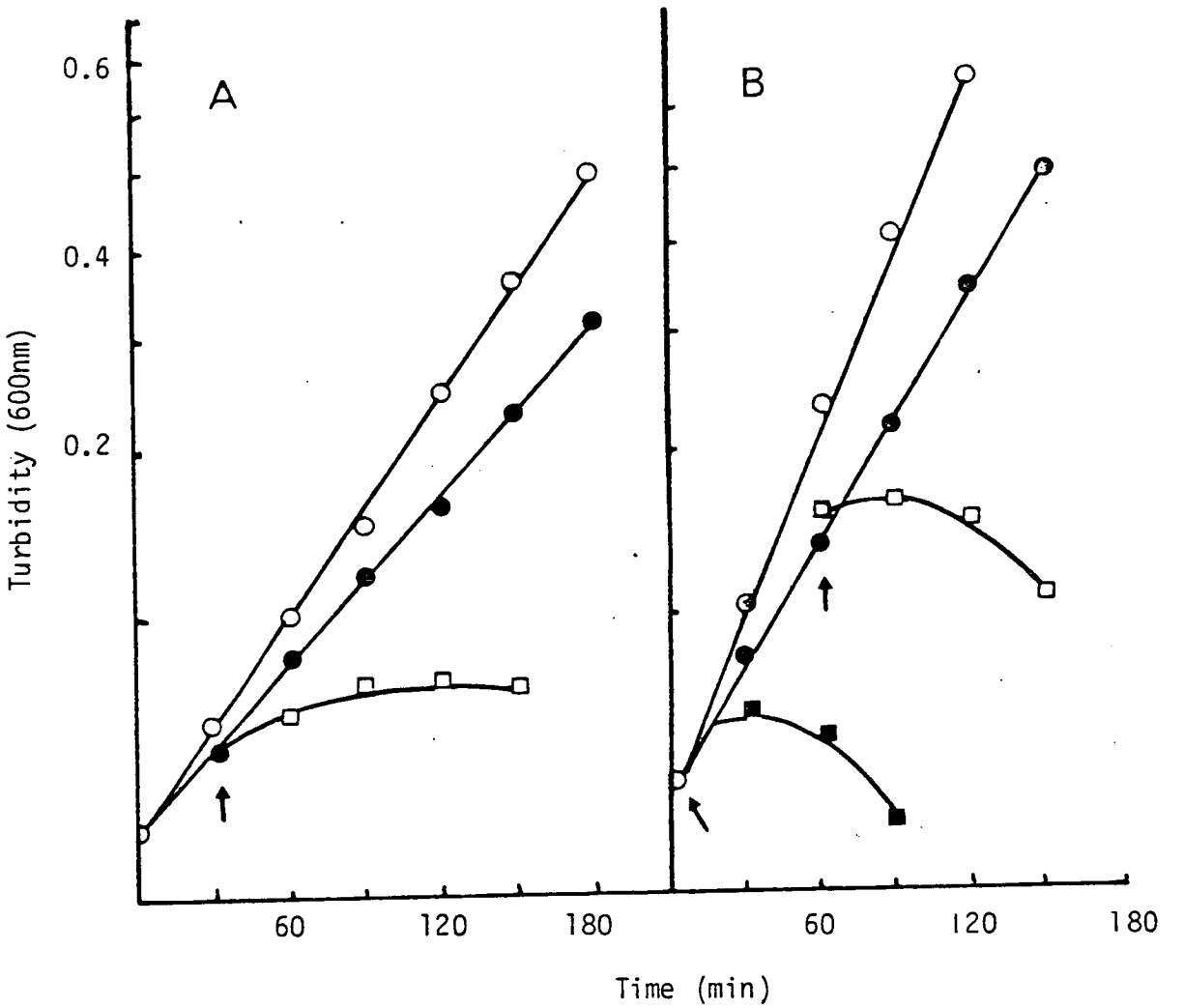


Figure 36. Effect of DCCD and CCCP on chloroquine inhibition of vegetative growth. Cells were grown in NB in the presence of DCCD (A) or CCCP (B) from zero time. (○), no additions; (●), 5ug ml^{-1} DCCD (A) or 0 5ug ml^{-1} CCCP (B); (□), 5ug ml^{-1} DCCD (A) or 0.5ug ml^{-1} CCCP (B) and chloroquine (500ug ml^{-1}). Chloroquine was added at the time indicated by the arrows.

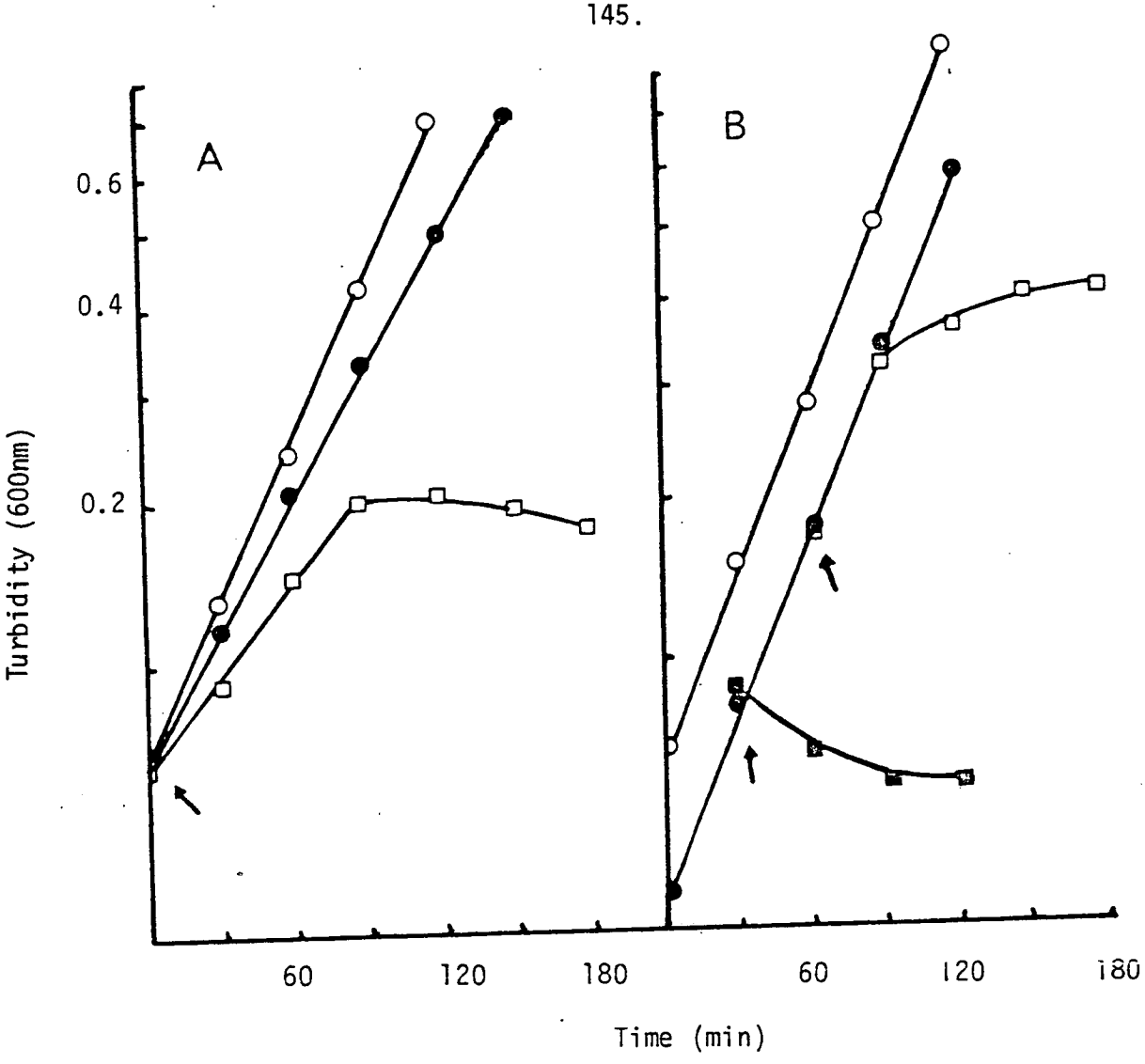


Figure 37. Effect of DNP and TCS on chloroquine inhibition of vegetative growth. Cells were grown in NB containing DNP (A) or TCS (B). (○), no additions; (●), 16ug.ml^{-1} DNP (A) or 0.75ug.ml^{-1} TCB (B); (□■), 16ug.ml^{-1} DNP (A) or 0.75ug.ml^{-1} TCS (B); and chloroquine (500ug.ml^{-1}). Chloroquine was added at the time indicated by the arrow.

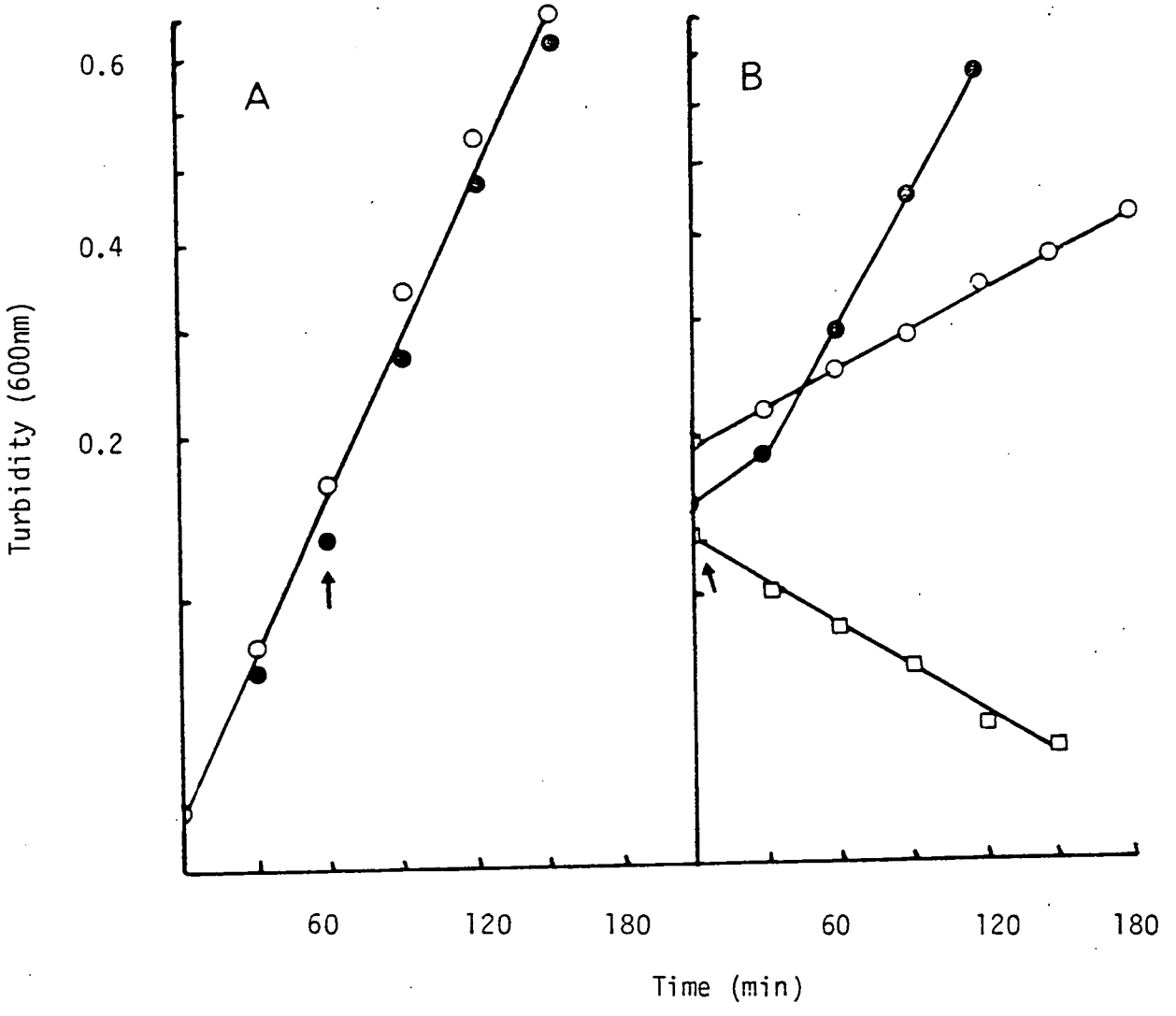


Figure 38. Effect of valinomycin on chloroquine inhibition of vegetative growth. Cells were grown in NB A, (○), 230 mM K Cl; (●), 0.1ug ml^{-1} valinomycin, 230 mM K Cl and chloroquine (500ug ml^{-1}). B, (○), 2mg ml^{-1} chloroquine (●), 2mg ml^{-1} chloroquine and 230 mM K Cl; (□), 2mg ml^{-1} chloroquine, 230 mM K Cl and 0.1ug ml^{-1} valinomycin. Chloroquine was added at the time indicated by the arrows.

differences in their energy metabolism. Therefore, addition of the metabolic inhibitors to outgrowing spores would not be expected to increase the inhibitory effect of a sublethal concentration of chloroquine. In fact, outgrowing spores were not sensitized to 100ug ml^{-1} chloroquine (a concentration of drug which inhibits outgrowth by about 20%) by any of the metabolic inhibitors used previously (Table 15). The concentration of chloroquine used (100ug ml^{-1}) had no effect on vegetative cells even in the presence of metabolic inhibitors. It is likely that vegetative cells are able to exclude, detoxify or counteract the effect of chloroquine through some metabolic process which is either not active during early outgrowth or active at a reduced level.

Novobiocin

Novobiocin, another preferential inhibitor of spore outgrowth (Gottfried et al., 1979) has ionized side groups (pK.a 4.3, 9.1) and, therefore, it is possible that the preferential inhibition is due to differential uptake. Again by finding the mic of novobiocin at different pH values it was shown that although at pH7 outgrowing spores were more sensitive to chloroquine than vegetative cells, at pH6 the mic had fallen and the preferential inhibition of spore outgrowth was not apparent (Table 18). It is unlikely that the modes of action of novobiocin (DNA gyrase inhibitor; Sanzey, 1979) and chloroquine (intercalation with DNA; Waring, 1970) that they inhibit precisely the same function in cells. Furthermore, results have been obtained which have shown that at pH7, where both inhibitors act preferentially towards outgrowing spores, germinated spores blocked by novobiocin during outgrowth do not develop further after washing and resuspension in medium containing chloroquine, but that spores blocked by chloroquine can, after washing continue outgrowth in the presence of novobiocin (Fig. 39).

Table 15. The effect of various agents on chloroquine inhibition of spore outgrowth.

Inhibitor (ug ml^{-1})	% Inhibition (observed)	% Inhibition (expected)*
Chloroquine (100)	12-22	-
2, 4 Dinitrophenol (16)	23-37	-
DCCD (5)	25-29	-
Rotenone (20)	26-31	-
CCCP (0.5)	14-21	-
Oxalate (1mg)	4-10	-
2,4 DNP Chloroquine	28-32	35 - 59
DCCD Chloroquine	50-60	37 - 51
Rotenone Chloroquine	47-63	38 - 54
Oxalate Chloroquine	22-26	16 - 32
CCCP Chloroquine	30-36	26 - 42

Spores were pre-germinated and grown out as described in the legend of Figure 29. Inhibitors were added at the same time. The results were the average of 4 experiments.

*Expected inhibition is the sum of % inhibition observed for each inhibitor.

Table 16. Inhibitory effect of Novobiocin at different pH values

pH	mic novobiocin [ug.ml^{-1}]	
	Outgrowth	Vegetative Growth
6	2 (16.6)*	2
7	6 (2)	7
8	7 (0.2)	9

For legend see Table 13.

*The figures in parentheses represent the percentage of uncharged novobiocin present.

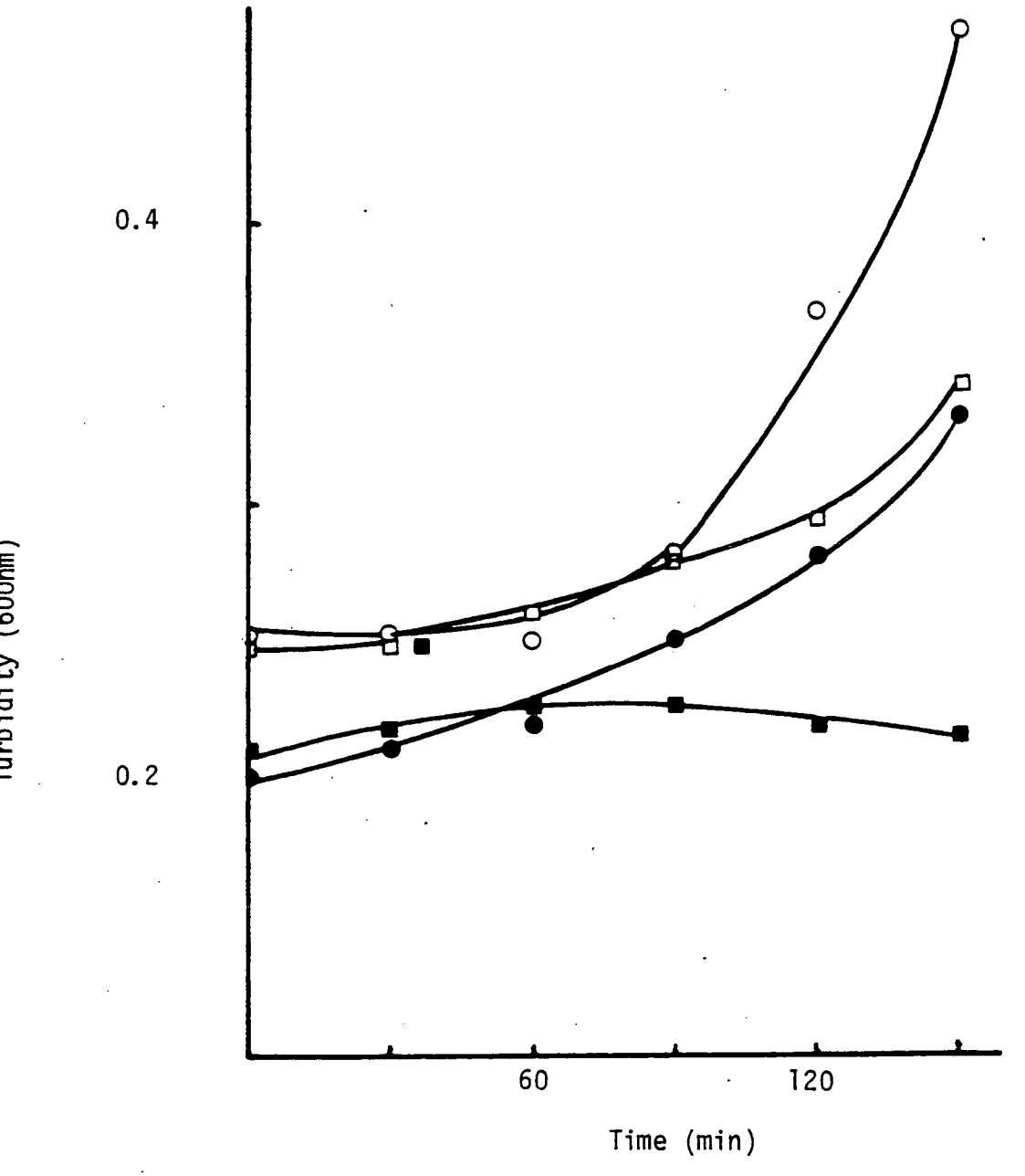


Figure 39. Temperal relationship between chloroquine and novobiocin inhibition of spore outgrowth. Pre-germinated spores were resuspended in NB containing either 500ug ml^{-1} chloroquine or 6ug ml^{-1} novobiocin for 1h, the cells were then centrifuged, washed with NB and resuspended in NB containing the appropriate drug. (○), blocked with chloroquine into NB; (●) blocked with novobiocin into NB; (□), blocked with chloroquine into novobiocin; (■), blocked with novobiocin into chloroquine.

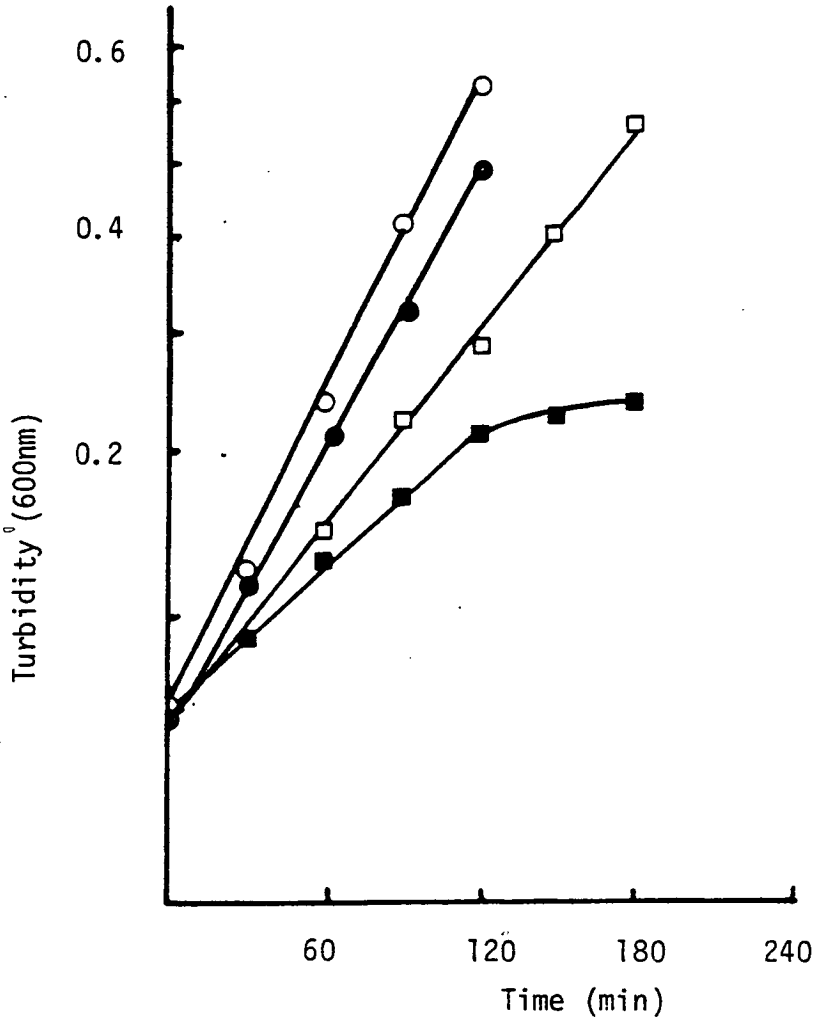


Figure 40. Effect of DNP on novobiocin inhibition of vegetative growth. Cells were grown in NB. (○), no additions; (●), 16ug ml^{-1} DNP; (□), 2ug ml^{-1} novobiocin; (■), 16 ug ml^{-1} DNP and 2ug ml^{-1} novobiocin. All drugs were added at zero time.

Novobiocin inhibition was also enhanced by metabolic inhibitors Figure 40 shows the enhanced inhibition of vegetative growth by 2ug.ml^{-1} novobiocin in the presence of 16ug.ml^{-1} DNP. Therefore it is likely that the preferential inhibition of spore outgrowth by certain inhibitors is not due to increased sensitivity or change in drug target during outgrowth, rather some aspect of energy metabolism affects the drug uptake (or metabolism) in some way.

Conclusions

The factors which influence the uptake of chloroquine by both outgrowing spores and vegetative cells have been examined.

The degree of ionization of a drug can influence its uptake and the more lipid soluble a molecule the easier it will penetrate the cell membrane (Franklin, 1973). Chloroquine has two ionizable amine groups of pKa8.1 and 10.1; as the pH of the medium approached the pKa 8.1 the mic of chloroquine for both outgrowth and vegetative growth dropped 5 fold and 50 fold respectively to 100ug ml^{-1}. In the case of outgrowth this correlates well with the percentage of monovalent and more lipophilic molecules present i.e. 11 to 55%, however in the case of vegetative cells the much larger drop in mic of 50 fold may indicate that some other factor apart from the lipophilic properties of the drug are involved in chloroquine uptake by vegetative cells. If outgrowing spores were more permeable to the divalent form of chloroquine one would not expect such a close correlation with the level of monovalent chloroquine present. The difference between sensitivity of outgrowing spores and of vegetative cells is unlikely to be due to the drug acting on a different or less sensitive target in the vegetative cells, otherwise the 10 fold difference in mic would be maintained

at different pH values, although the mic would still change. Also, there was no change in the pH of the medium in which outgrowth occurred and therefore it is unlikely that this influenced the mic (data not shown) While it is possible that teichoic acids, which are only present in vegetative cells and late outgrowing spores could prevent entry of charged antibiotics there was no enhanced inhibition of vegetative cells grown under conditions in which teichoic acids may have not been synthesized. Teichuronic acids, however, synthesized in place of teichoic acids, have a comparable charge and so it is possible that they too could prevent entry of chloroquine to the cell. However, it has been suggested that streptomycin, another positively charged molecule, binds to teichoic acids to generate a high and localized drug concentration and so can more effectively disrupt the permeability barrier of the membrane (Franklin & Snow, 1975). If chloroquine were binding to teichoic acid it might be expected that vegetative cells would be more sensitive to chloroquine than outgrowing spores. Moreover, chloroquine inhibition was independent of cell concentration (Chapter 4) and it is unlikely that this would be the case if chloroquine were binding to teichoic acids.

The changes in the lipid components and the lipid/protein ratio during outgrowth (Bulla et al., 1975; Ellar, 1978) again could influence entry of drugs into the cell, but this was not evident in the experiments using alkyl-p-hydroxybenzoates and it was found that there were no significant differences in mic between outgrowing spores and vegetative cells with a range of molecules of differing lipid solubility and charge. Furthermore, phenethyl alcohol, ethanol and polymyxin, all molecules that disrupt membrane structures (Hamilton, 1971) did not increase cell sensitivity to chloroquine, although this effect has been reported for streptomycin and

acriflavin in E. coli (Anand et al., 1960; Jackson & De Moss, 1965). Therefore it is likely that any differential permeability of outgrowing spores to chloroquine is not due to differences in cell membrane per se.

However, a range of inhibitors which affect energy metabolism, in particular the electron transfer chain and the maintenance of the membrane potential, enhanced chloroquine inhibition markedly in vegetative cells but not outgrowing spores. It is unlikely that the enhanced inhibition is due to an effect on the cell membrane, because molecules which disrupt the membrane do not have the same effect. Furthermore, rotenone and oxalate did not enhance chloroquine uptake in minimal medium. In this medium the cells obtain most of their energy from the glucose present by glycolysis and the TCA cycle is functioning as an anabolic pathway; oxalate will be ineffective since the enzymes it inhibits (succinate dehydrogenase and malate dehydrogenase) will be repressed (Hanson & Cox, 1967; Weber & Broadbent, 1975). The same explanation holds for rotenone which only acts on ATP synthesis via the respiratory chain oxidation of NADH generated largely in the TCA cycle. When cells are fermenting glucose the NADH is reoxidized in the final steps of the fermentation sequence and ATP is generated at the substrate level. If these compounds were acting on the membrane one would not expect different results dependent on medium composition.

The metabolic function linked to energy generation in vegetative cells presumably prevents entry of chloroquine into the cell, or increases efflux of the drug or detoxifies the drug, and this function is not present, or is less active in outgrowing spores. The results obtained with novobiocin may indicate that the phenomenon of preferential inhibition of spore outgrowth is not due to a specific change in drug targets as has been

suggested by Gottfried et al. (1979) but rather to exclusion or dextoxification of drugs by vegetative metabolic processes that in general are absent from outgrowing spores. There are two particularly pertinent inhibitor studies which support this hypothesis. Staal and Hoch (1972) have isolated two classes of streptomycin/dihydrostreptomycin resistant B. subtilis mutants which are sensitive to the drugs during outgrowth and sporulation, both genes coding for this resistance were mapped in loci away from the normal ribosomal change which normally confers resistance to streptomycin. Resistance to the drugs during vegetative growth was only observed in minimal medium with glucose but not in rich medium; ribosomes from both wild type cells and resistant mutants bound streptomycin to the same extent and it is likely that the resistance is due to a permeability barrier in the mutants (Staal & Hoch, 1972). The second study concerned ethidiumbromide-resistant mutants of B. subtilis. Bishop and Brown (1973) have shown that the resistance to ethidium bromide was also only present in vegetative cells and was lost during sporulation and early outgrowth. The resistance during vegetative growth was conditional and was only apparent at 37°C and 45°C but not 30°C, the authors suggested that some cell component possibly in the membrane was responsible for the resistance to ethidium bromide and that this was not present or active during sporulation and early spore outgrowth. (Bishop & Brown, 1973). Both cases are very like that of chloroquine inhibition i.e. a possible permeability barrier present only in vegetative growth but not during sporulation or outgrowth and the observation that the streptomycin resistance was medium dependent may indicate that some aspect of cell metabolism is involved in the resistance.

The possible nature of this function and the significance of the

enhanced inhibition of chloroquine by various metabolic inhibitors is discussed further in the next chapter in the light of experiments on the actual uptake of chloroquine by outgrowing spores and vegetative cells.

CHAPTER 7

Uptake of chloroquine by outgrowing spores and vegetative cells

Introduction

There are no published studies of chloroquine uptake by bacteria. However, the results of work on chloroquine uptake by eukaryotic cells have been reported several times. Red blood cells containing malarial parasites concentrate chloroquine from the surrounding medium to high levels, and various extents of concentration relative to non-infected erythrocytes have been reported ranging from 500:1 to 40:1 (Fitch, 1969; Polet & Barr, 1968); resistant parasites concentrated less drug (Fitch, 1969). The initial uptake of the drug was energy independent and not due to active transport but there was apparently a later phase that did .. depend on energy (Polet & Barr, 1968). However, in Chang liver cells chloroquine uptake was reduced by metabolic inhibitors and low temperatures (Polet, 1976).

It appears then that there exist a variety of mechanisms by which chloroquine can be concentrated by cells, but the significance of these studies to understanding bacterial uptake of the drug is limited since in several eukaryotic cases lysosomes appear to be involved (Polet, 1976; Homewood et al., 1972) and these organelles are absent in prokaryotes.

In the previous chapter evidence was presented which may indicate that outgrowing spores are more permeable to chloroquine than vegetative cells and that vegetative cells can be made sensitive to low concentrations of chloroquine by treatment with a variety of metabolic inhibitors. It is possible, therefore, that the preferential inhibition of spore outgrowth is the result of a greater accumulation of the drug by spores due to the absence of an active mechanism (related to electron transport functions) needed to exclude the drug. In order to test this hypothesis it is necessary to measure the actual amounts of the drug in spores and vegetative cells.

Uptake of chloroquine

If early outgrowing spores are more permeable to chloroquine than vegetative cells they should accumulate more chloroquine than an equivalent number of late outgrowing spores (which are resistant to 500ug ml^{-1} chloroquine) and vegetative cells. The time course of [^{14}C] - chloroquine accumulation by early and late outgrowing spores and vegetative cells is shown in Figure 41. The overall pattern of accumulation was the same for all three cell-types; initial uptake of chloroquine was very rapid, reaching saturation levels within 2 min, although early outgrowing spores accumulated the drug more slowly than the other cells. After this there was little net accumulation of chloroquine. Nevertheless, there were major differences in the concentration of label accumulated by the three cell-types and early and late outgrowing spores retained, after five minutes exposure, three times and eight times more chloroquine respectively than an equivalent number of vegetative cells. However, there are large changes in cell volume during spore outgrowth (Hitchins *et al.*, 1963) and it is necessary to account for these changes in more accurately assessing the differential uptake of chloroquine by cells.

The outgrowing *B. subtilis* spore has a length of 5.8um and a diameter of 0.88um by first septation (Umeda & Amako, 1980), if one assumes the cell is a cylinder, 10^8 cells would have a volume of 0.35mm^3. This volume is four-times greater than that of a germinated spore (Hitchins *et al.*, 1963), therefore, 10^8 germinated spores (early outgrowing spores) would have an approximate volume of 0.087mm^3. A vegetative *B. subtilis* cell can be considered to be half the size of a late outgrowing spore (at first septation) and 10^8 vegetative cells would have a presumptive volume of 0.175mm^3. The relative accumulation of chloroquine by early

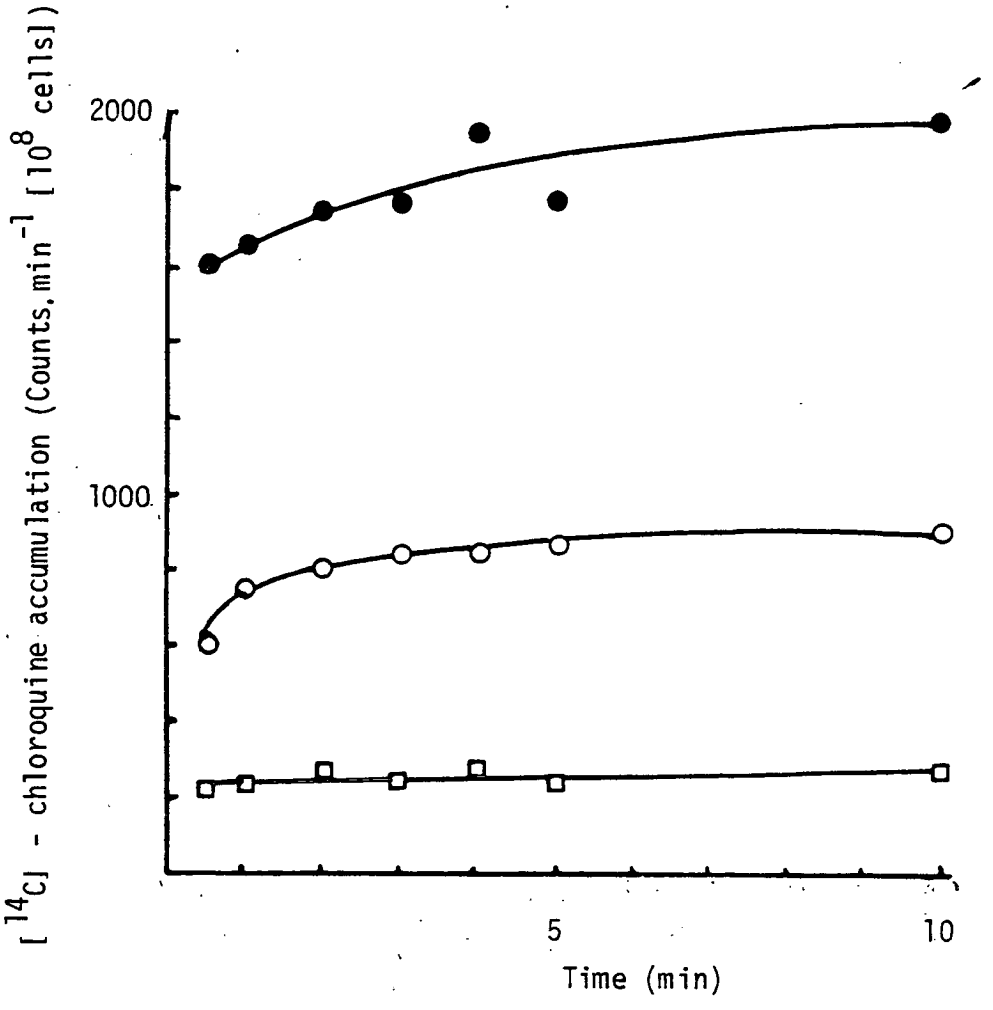


Figure 41. Kinetics of chloroquine accumulation. Outgrowing spores and vegetative cells were incubated with [^{14}C] - chloroquine in NB. Accumulation of chloroquine was measured as described in the methods. (○), early outgrowing spores; (●), late outgrowing spores; (□), vegetative cells.

and late outgrowing spores and vegetative cells corrected for volume in shown in Table 17. Late outgrowing spores and vegetative cells accumulated 53% and 86% less chloroquine respectively than early outgrowing spores. Therefore, the preferential inhibition of outgrowth by chloroquine may be explained by differential accumulation of the drug, assuming the calculations of cell volume are accurate. The results presented above refer to experiments in which no cold carrier chloroquine was present, and addition of label alone gave a concentration in the medium of 1.7ug ml^{-1} chloroquine. It is possible that in the presence of higher concentrations of chloroquine different results would be obtained and this possibility is examined below.

In the presence of 500ug ml^{-1} chloroquine the accumulation in 5 min of [^{14}C]- chloroquine by early and late outgrowing spores and vegetative cells was reduced by 76%, 79% and 70% respectively (i.e. counts. min^{-1}) and the pattern of uptake was not altered (data not shown). Therefore, there was an overall reduction in the accumulation of the labelled drug when 500ug ml^{-1} chloroquine (corresponding to approximately 1mM) was present which applied equally to each stage of growth examined. From these results it was possible to estimate an approximate internal concentration of chloroquine at each growth stage by comparing counts.min^{-1} [^{14}C] - chloroquine per mm^3 of medium to counts.min^{-1} [^{14}C] - chloroquine per mm^3 of cell volume. Early outgrowing spores, late outgrowing spores and vegetative cells concentrated chloroquine approximately 57, 27 and 8 fold respectively from a medium in which it was present at 500ug ml^{-1} (i.e. 1mM), corresponding to an internal concentration of 57, 27 and 8mM. It was important at this stage to check that the radioactive label accumulated by cells corresponded to chloroquine before any conclusions could be drawn.

Table 17. Comparative accumulation of chloroquine by outgrowing spores and vegetative cells

Stage of Growth	Volume (10^8 cells) mm^3	Labelled Chloroquine* Retained (counts.min^{-1})	Specific accumulation (counts.min^{-1})	Relative Accumulation %
Early Outgrowth (60 min)	0.087	830	9485	100
Late Outgrowth (150 min)	0.35	1577	4506	47
Vegetative Growth	0.175	236	1348	14

*Average of three measurements corrected for added label. Uptake and accumulation of chloroquine was measured as described in the methods. Specific uptake is counts retained in 5 min divided by cell volume i.e. counts per mm^3. Relative accumulation the percentage specific accumulation compared to that of early outgrowing spores.

Purity of chloroquine

In order to check that the label accumulated by cells was chloroquine, various samples were analysed by thin layer chromatography (TLC). Non-radioactive chloroquine and labelled samples of chloroquine were compared with the [^{14}C] - chloroquine remaining in the medium after removal of cells and the label concentrated by the cells. In the latter case chloroquine was recovered from late outgrowing spores by pelleting them as described in the methods and resuspending the cells in 0.5N Na OH in half strength NB containing 10ug ml^{-1} chloroquine to minimize binding to glassware. The mixture was left at 20^{o}C for 30 min and cells removed by centrifuging through a silicone oil mixture in which more than 90% of the label was removed from the cells by this treatment. The supernatant (0.5ml) was removed and dried by rotary evaporation under vacuum and made up to 50ul in distilled water. The remaining three samples were made 0.5N with respect to Na OH in half strength NB, dried as described above and made up to 50μl in distilled water before application to the TLC plate. After chromatography the chloroquine was visualized as fluorescent areas under a long-wave ultra-violet light source (366nm) and all samples, including a chloroquine control not subjected to Na OH and drying, appeared as a single spot with an Rf value of 0.25. By assaying sections of the thin layer plates for radioactivity it was found that the great majority of the label was in the region of the chromatogram in which chloroquine was detected as a flourescent spot and there were no other obvious peaks of radioactivity (Fig. 42). Therefore, it is likely that the radioactivity measured in cells corresponded to chloroquine and furthermore that chloroquine was not modified in late outgrowing spores that show greater resistance to the drug.

Mechanism of chloroquine concentration

Chloroquine is concentrated from the medium by outgrowing spores and vegetative cells and it is important to establish the nature of the mechanism by which chloroquine is concentrated from the medium.

Bacteria can concentrate drugs from the medium by passive diffusion or by facilitated transfer. In the first case, at equilibrium an ionized drug such as chloroquine may distribute unequally across the cell membrane because of a Donnan equilibrium, a pH difference across the membrane, or due to the drug binding to a target site inside the cell. In the second case the drug is concentrated actively in the cell by transport sites which may show a high degree of specificity. There are a number of ways to distinguish between the two possibilities: at low temperatures the accumulation of drug by passive diffusion would be unaffected whereas uptake would be reduced if the drug were concentrated by facilitated transfer.

When accumulation of chloroquine by outgrowing spores and vegetative cells was measured at $4^{o}C$, early outgrowing spores concentrated 22% less chloroquine, whereas late outgrowing spores and vegetative cells concentrated 42% and 460% more chloroquine than at $30^{o}C$. Therefore two conclusions can be drawn: firstly, chloroquine is concentrated in cells by passive diffusion; secondly, it is likely that late outgrowing spores and vegetative cells are actually excluding the drug by some energy-dependent mechanism. The last statement is supported by the results presented in Chapter 6, in which inhibitors of energy metabolism increased the inhibitory effect of chloroquine in vegetative cells but not outgrowing spores.

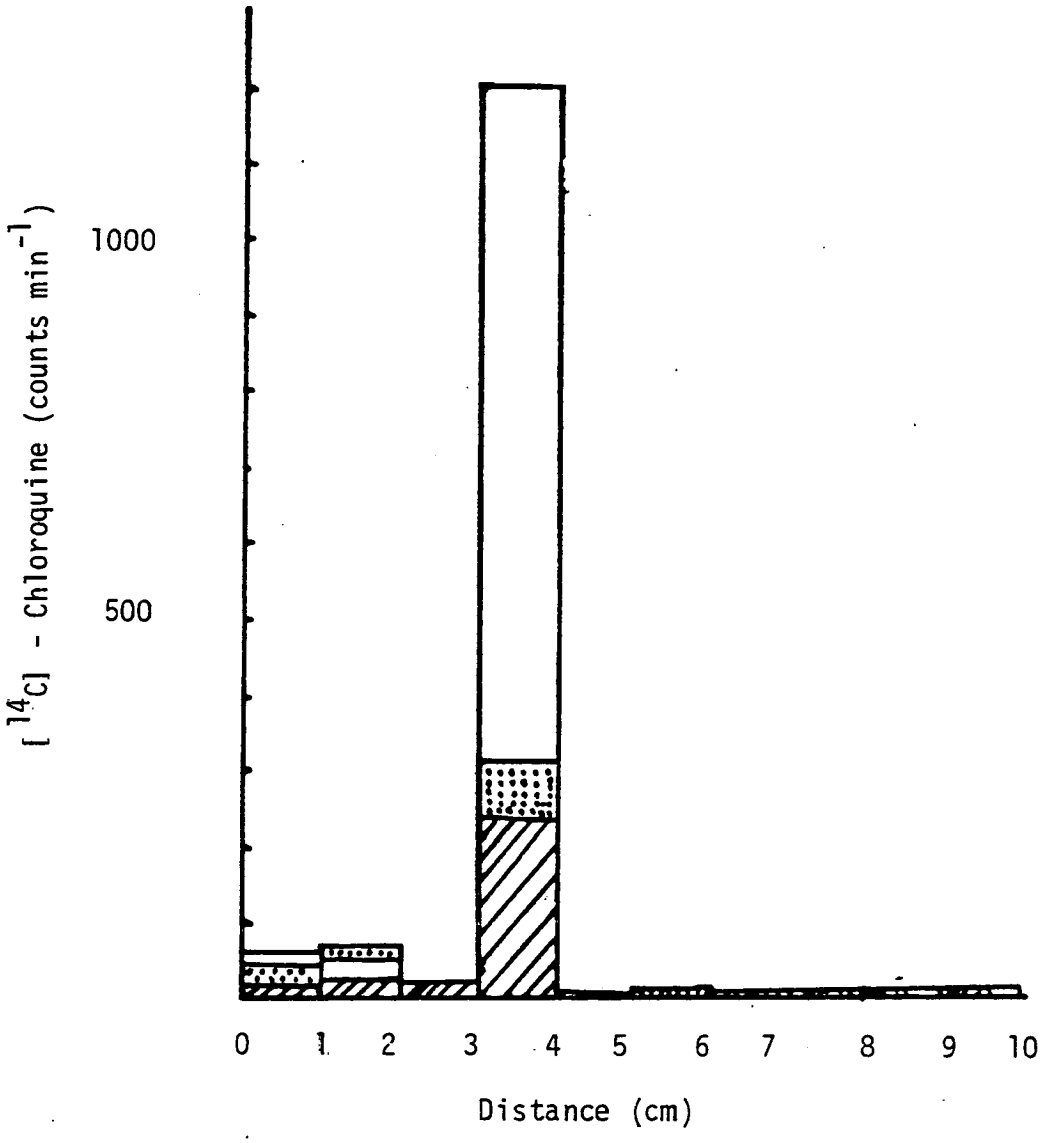


Figure 42. TLC analysis of chloroquine. Samples of [^{14}C] - chloroquine were prepared as described in the text and run for 2h on silica gel TLC plates using a methanol : acetone : diethylamine (10:10:03, V/V/V) solvent system. Sections of TLC plate (1x1cm) were suspended in triton/toluene scintillant before radioactivity was measured. (:::), [^{14}C] - chloroquine; ( ), supernatant chloroquine; (////), chloroquine in cells.

Do these inhibitors increase uptake of chloroquine by vegetative cells? The results of experiments in which the uptake of chloroquine was measured in vegetative cells treated with various inhibitors are presented in Table 18. If one considers the results obtained when no extra chloroquine was present, the inhibitors appeared to fall into three groups. Ionophores i.e. CCCP and 2, 4-DNP decreased accumulation of the drug by 22% and 14% respectively, whereas DCCD (an ATPase inhibitor) increased uptake by 210% and oxalate (a dehydrogenase inhibitor) by 23%. The surface active agents i.e. phenethylalcohol and ethanol had little effect. These results may indicate that the ionophores, DCCD and oxalate are affecting different aspects of chloroquine uptake. The results obtained when 500ug ml^{-1} chloroquine was present support this idea. The ionophores CCCP and 2, 4-DNP increased accumulation by 350% and 179% respectively, but additional chloroquine did not affect the level of chloroquine accumulated in the presence of DCCD (207%) seen when no extra chloroquine was present. Again the surface active agents did not affect uptake to any significant extent. In summary DCCD increased the accumulation of chloroquine in a way that was independent of the chloroquine concentration, whereas ionophores decreased accumulation at low levels of chloroquine and increased accumulation at higher concentrations of chloroquine. Since surface active agents did not significantly affect chloroquine accumulation in either case it is doubtful if the other inhibitors were increasing chloroquine uptake by perturbing the membrane per se. The concentration of chloroquine by outgrowing spores was not altered by any of these inhibitors.

It is not clear whether the ionophores at low chloroquine concentration were preventing entry of the drug or were increasing efflux

Table 18. Effect of various inhibitors on chloroquine uptake in vegetative cells.

Inhibitor (ug ml^{-1})	Specific chloroquine accumulation	
	No added[1] cold chloroquine	Unlabelled[2] chloroquine (500ug ml^{-1})
CCCP (0.5)	1050	1622
CCCP (0.75)	1068	ND
2, 4-DNP (16)	1159	737
DCCD (5)	2822	851
Oxalate (1mg ml^{-1})	1662	605
Phenethyl alcohol (1mg ml^{-1})	1404	426
Ethanol (1%)	1303	419
NONE	1348	411

* counts.ul^{-1} internal volume, determined in triplicate as described earlier.

1 radioactive chloroquine only

2 radioactive chloroquine and 500ug ml^{-1} chloroquine

ND not done

(there is an equilibrium between the drug entering and leaving the cell). In an experiment in which CCCP was added at the same time as [^{14}C] - chloroquine and accumulation followed with time, the initial uptake of the drug was the same as untreated cells but after 10 min net accumulation of the drug was reduced by 13%. Therefore, it is probable that the ionophores increased efflux of the drug at low chloroquine concentrations, but when higher concentrations of chloroquine were present net uptake was increased; this is possibly related to a decreased efflux of the drug due to the influence of chloroquine. Since DCCD added at the same time as chloroquine increased accumulation of the drug with time, no conclusions can be drawn as to whether DCCD is influencing the uptake or efflux of chloroquine.

Nevertheless, the metabolic inhibitors increased the internal chloroquine concentration of vegetative cells to that found in outgrowing spores. Therefore, the preferential uptake of chloroquine by outgrowing spores is probably due to the absence of a mechanism in outgrowing spores to either decrease influx or increase efflux of chloroquine. Furthermore, that this is related to the energy generating system of cells possibly <u>via</u> the internal pH of cells or the membrane potential. This is investigated further below.

<u>Effect of pH on chloroquine uptake</u>

Chloroquine is concentrated in cells by passive diffusion due to either a Donnan equilibrium across the membrane, a pH difference across the membrane by or binding to a target inside the cell. Chloroquine can bind in cells by intercalating with DNA (Cohen & Yielding, 1965b), however the very high concentrations of chloroquine accumulated by the cells (8 to 57mM)

are not compatible with the amount of DNA in the cells i.e. there would be insufficient DNA to account for the amount of drug bound. It would be difficult to distinguish between drug accumulation by Donnan equilibrium or a pH difference across the membrane. Results presented in Chapter 6 may indicate that cells are more permeable to the monovalent chloroquine than the divalent ion. It is possible that on entering a cell the internal pH is sufficient to allow chloroquine to ionize to the divalent form of the drug which cannot pass through the membrane, and this would be equivalent to the drug being bound to a specific target inside the cells.

Accumulation of chloroquine by outgrowing spores and vegetative cells varied with pH (Fig. 43), at pH 6 (where the majority of chloroquine is in the divalent form) uptake was reduced; as pH increased accumulation of chloroquine also increased in a manner consistent with differential uptake of monovalent and divalent chloroquine. In late outgrowing spores and vegetative cells, however, the increased accumulation of chloroquine between pH 7 and 8 was less than that in early outgrowing spores. A number of conclusions can be drawn from these experiments. First, cells are less permeable to the divalent form of chloroquine than the monovalent form, much of the binding seen at pH 6 may be external as dormant endospores bind about 14% of label accumulated by early outgrowing spores in 2 min at pH 7, although there is no indication to which site in or on the dormant spore this label is bound. Therefore, it is possible that chloroquine is bound inside cells as the divalent ion. Secondly, the observation that early outgrowing spores concentrate relatively more chloroquine at pH 8 than late outgrowing spores and vegetative cells, may be due to either decreased permeability to monovalent chloroquine in late outgrowing

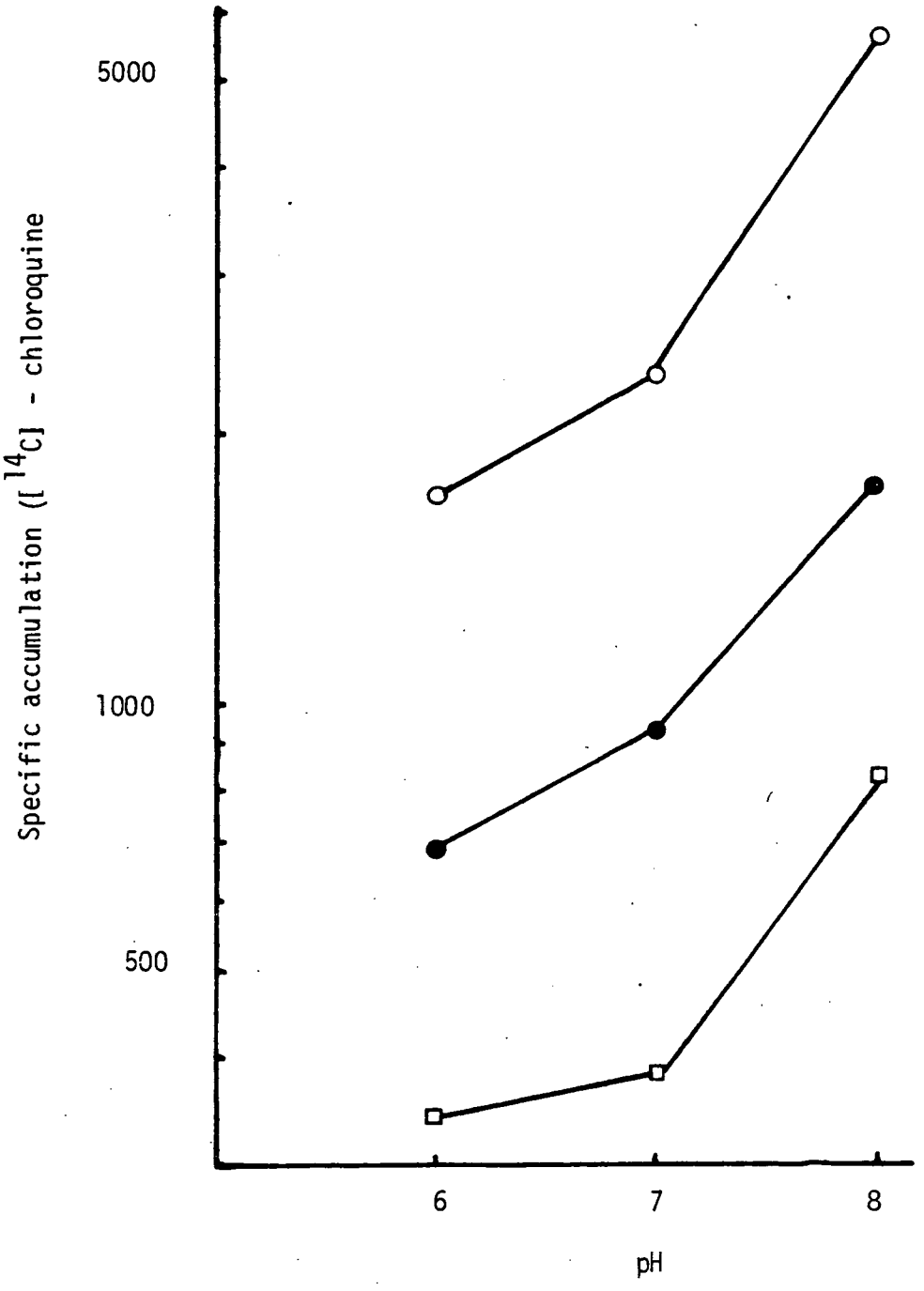


Figure 43. Effect of pH on chloroquine uptake. Outgrowing spores and vegetative cells were suspended in NB adjusted to the required pH with Na OH or HCL, containing chloroquine (500ug ml^{-1}), before accumulation was measured (○), early outgrowing spores; (●), late outgrowing spores (□), vegetative cells.

spores or vegetative cells or a difference in their ability to bind the ion.

It is now possible to formulate an hypothesis to explain the differential accumulation of chloroquine by early outgrowing spores and vegetative cells;if the internal pH of early outgrowing spores is lower than that of the resistant forms (i.e. late outgrowing spores and vegetative cells) more chloroquine would be found in the cytoplasm since monovalent chloroquine would pass out of the membrane phase into a more acid cytoplasm with production of the doubly protonated form, insoluble in lipid and incapable of passing back into the membrane. In the less acid cytoplasm of the resistant forms less chloroquine would be converted to the divalent form and so more would be able to pass out of the membrane. A similar situation may exist in the lysosomal organelle in eukaryotic cells in which the low pH of the lysosome is though to be the reason for its ability to concentrate chloroquine (Homewood et al., 1972; Helenius et al., 1980).

If this were the case it might be expected that the rate of excretion of chloroquine from early outgrowing spores and vegetative cells would differ and this is examined in the next section.

Efflux of chloroquine from cells

The inhibitory effect of chloroquine can be reversed by washing the cells free of the drug (Chapter 4), therefore chloroquine can diffuse out of cells and is not irreversibly bound. When outgrowing spores and vegetative cells were incubated with [^{14}C] - chloroquine, collected by membrane filtration, and resuspended in medium without chloroquine, the drug was lost from the cells (Fig. 44). The rate of loss from outgrowing spores and vegetative cells was similar, however, the amount of drug retained after filtration differed; early outgrowing spores retained 24% of [^{14}C] - chloroquine present before filtration and washing whereas late outgrowing spores and vegetative cells retained only 8-9% label. It is possible that this difference was due to the 14% of label that appeared to be bound to non-germinated spores mentioned in the last section. Therefore, the experiment was repeated but the cells were resuspended in NB containing 500ug ml^{-1} chloroquine, thereby measuring the exchange of chloroquine between the cells and medium (Fig. 45). Again the rate of exchange was similar in early outgrowing spores and late outgrowing spores and vegetative cells but in this case early outgrowing spores retained about 5 times more radioactive chloroquine than the resistant cells i.e. there was less exchange of chloroquine between intracellular and extracellular chloroquine in early outgrowing spores. When CCCP was present in the resuspension medium after the filter wash vegetative cells exchanged about two-fold less chloroquine. Therefore, it is possible that the ionophore increases the chloroquine bound in cells by decreasing the internal pH. However, when vegetative cells were treated with CCCP before resuspension or if they were treated with CCCP before resuspension and CCCP was present in the resuspension mixture the decreased exchange of chloroquine was not observed (data not shown) and it is difficult to assess the relevance of these

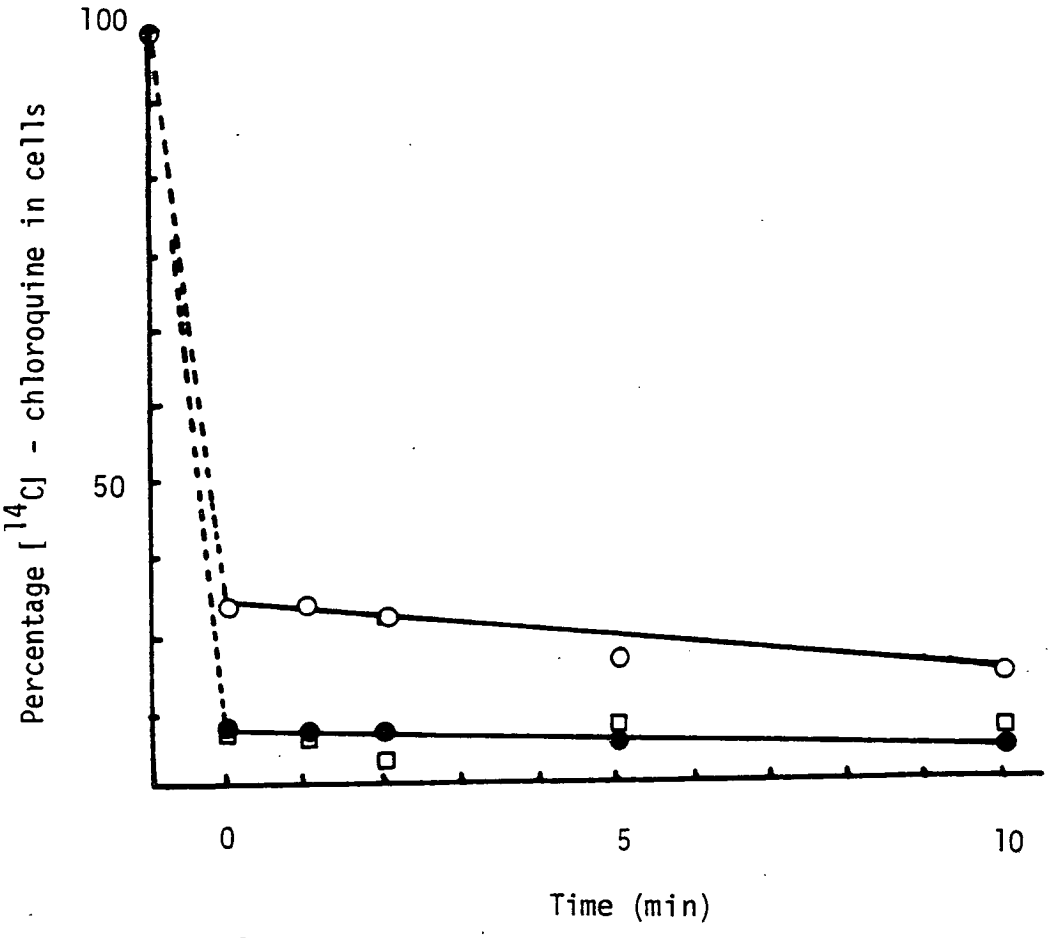


Figure 44. Loss of [^{14}C] - chloroquine from cells. Outgrowing spores and vegetative cells were incubated with radioactive chloroquine for 5 min. The pre-loaded cells were collected by membrane filtration, washed with NB containing 2ug ml^{-1} chloroquine and resuspended in NB [^{14}C] - chloroquine retention was followed as described in the methods. (○), early outgrowing spores; (●), late outgrowing spores; (□), vegetative cells. The broken line represents chloroquine retained before washing.

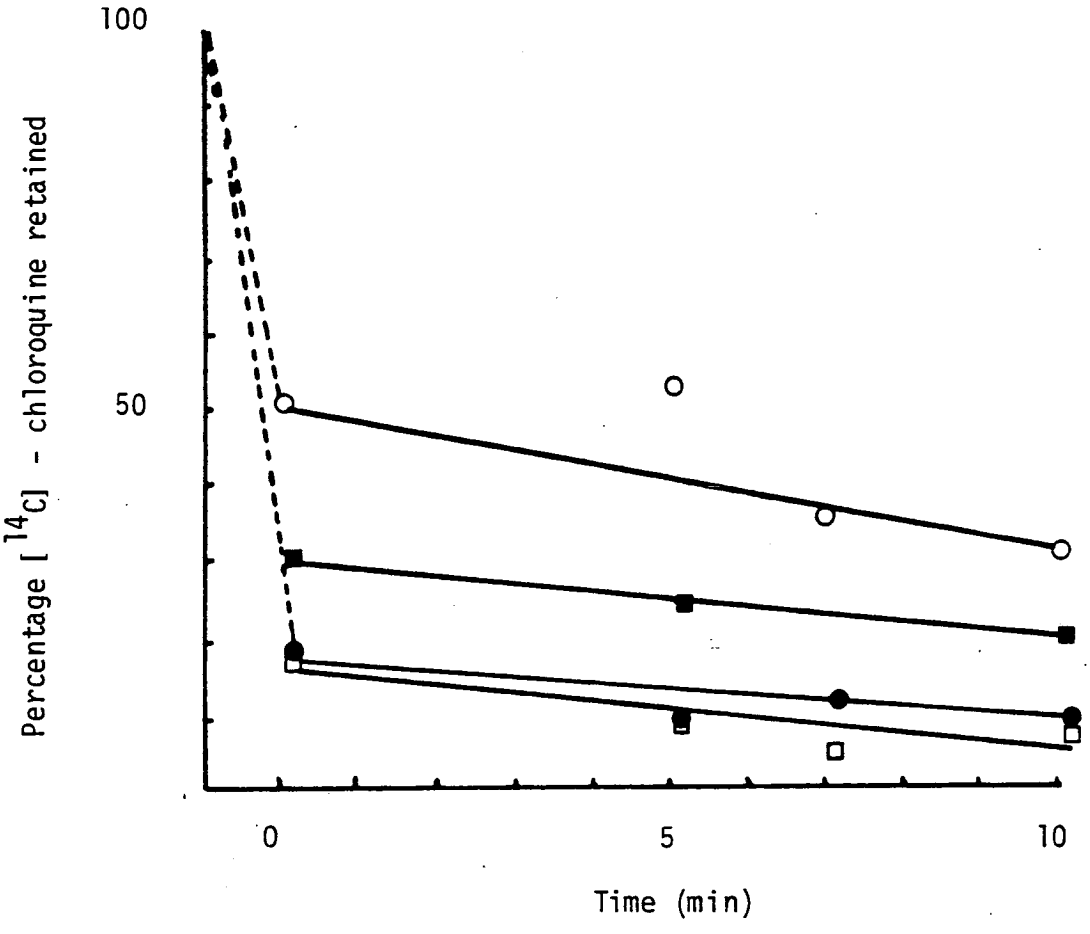


Figure 45. Exchange of intracellular and extracellular chloroquine. Cells were pre-loaded and washed as described in the legend of Figure 44 and resuspended in NB containing 500ug ml^{-1} chloroquine. Retention of radioactive chloroquine was measured as described previously in the methods section (○), early outgrowing spores; (●), late outgrowing spores; (□), vegetative cells; (■), vegetative cells and 0.5ug ml^{-1} CCCP. The broken line represents chloroquine retained before washing.

observations.

Overall it appears that early outgrowing spores bind more chloroquine than resistant cells and this is compatible with the hypothesis stated earlier. If the internal pH of outgrowing spores is lower than vegetative cells other amines would be taken up preferentially assuming that their pK value was such that they would ionize inside cells. A number of amines were tested for their ability to affect outgrowth and vegetative growth and methylamine (pK_a10.6) was found to inhibit outgrowth at a level which inhibited vegetative growth by only 60%. It is interesting to speculate that outgrowing spores accumulate more methylamine than vegetative cells because of a low internal pH, indeed methylamine and other amines are used to determine pH differences across cell membranes (Schuldiner et al., 1972).

Conclusions

In Chapter 6 it was suggested that the preferential inhibition of spore outgrowth by chloroquine was due to the ability of vegetative cells to detoxify or exclude the drug by an energy-dependent mechanism. Measurement of the concentrations of chloroquine accumulated by outgrowing spores and vegetative cells supported the latter suggestion that vegetative cells exclude the drug. However, a number of assumptions have been made which will influence the actual calculations of the amount of chloroquine concentrated by cells. The first assumption is that there will be little if any supernatant fluid bound in the cell pellet after centrifugation through the silicone oil mixture, it is reasonable to expect such a system to remove most of the supernatant and in experiments in which dextran blue was mixed with the cell suspensions before centrifugation there was no detectable dextran blue in the pellet (data not shown). Throughout these experiments any [^{14}C] - chloroquine bound by cells has been considered to be in the cytoplasm, although this may not be the case since dormant spores accumulated a significant level of label. It is difficult to determine to which part of the spore this binding occurs e.g. to the spore coat, matrix or the cytoplasm. The third most obvious area for large errors is in the assumptions made in calculating the cell volumes and at best these can only be considered approximate. The volume assumed for the spore is probably too large, as it does not allow for the dense spore coat. In calculating the volume of vegetative cells it was assumed that all the cells would be one size but this is not the case as cell size will range from one unit up to two units as the cell grows and divides. It is possible that spores accumulate more chloroquine and vegetative cells less, than the figures reported here.

Both vegetative cells and outgrowing spores concentrated chloroquine quickly to high levels (8 and 57 mM respectively) by passive diffusion since uptake was not prevented by low temperatures (4°C) or metabolic inhibitors. Furthermore, most drugs which are actively concentrated in cells by facilitated transfer are normally analogous of natural cellular metabolites but there was no evidence that chloroquine mimics such a metabolite (Chapter 4). It is unlikely that all of the binding of chloroquine in cells is to its supposed target i.e. DNA, since the spore contains only nanogram amounts of DNA, insufficient to bind all of the drug. Furthermore, late outgrowing spores which bind less chloroquine than early outgrowing spores have more DNA since the early outgrowing spore has a single chromosome which replicates as outgrowth proceeds (Strange & Hunter, 1969).

Is it possible to account for the large concentration of chloroquine in cells if one assumes that the cell membrane is impermeable to doubly protonated chloroquine but permeable to the monoprotonated form. In such a situation the monoprotonated molecule could pass into the cytoplasm and if the cytoplasm had a suitable internal pH (i.e. 6-8) the drug would ionize to the divalent form and so be effectively bound in the cell. Some evidence was provided for this hypothesis, at pH6 where the majority of the chloroquine molecules are monovalent less drug was accumulated by cells. However, there appeared to be a large amount of chloroquine concentrated at pH 6.0, since the doubly-protonated form of chloroquine is thought to be the active form and will bind to macromolecules (Cohen & Yielding, 1956; Parker & Irvin, 1952) it is possible that much of this label is bound extracellulary. Aside from the actual measurement of chloroquine accumulated at various pHs, it was shown earlier (see Chapter 6)

that the mic of chloroquine for outgrowth varied with pH such that there was a direct relationship between the mic and levels of monoprotonated chloroquine present i.e. about 50ug ml^{-1} monoprotonated chloroquine at pH 6, 7 and 8. Therefore it is likely that much of the binding at pH6 has no relationship to the actual physiological levels of penetrating chloroquine, rather the drug is binding extracellularly.

The effect of the various inhibitors on chloroquine uptake in vegetative cells was particularly interesting. DCCD (an ATPase inhibitor) increased accumulation of chloroquine irrespective of the extracellular chloroquine concentration. However, the H+ ionophores only increased chloroquine accumulation when 500ug ml^{-1} was present and at low chloroquine concentrations (1.7ug ml^{-1}) actually decreased accumulation, probably by increasing efflux of the drug.

If one considers the membrane potential across the cell membrane it is made up of two components, a gradient of pH and an electrical potential with the inside more alkaline and more electronegative compared to the outside. These gradients lead to a force on protons extruded by the respiratory chain, "the proton motive force", consisting of the sum of the two above components.

$$\Delta p = \Delta\Psi - Z\,\Delta pH$$

where; Δp is the proton motive force in electrical units; $\Delta\Psi$ is the difference in electrical potential across the membrane; $Z = 2.3RT/F$ and has a value of 60mV in the biological range; and ΔpH is the pH difference across the membrane (Harold, 1972). In B. subtilis, ΔpH at external pH values around pH 7-8 is negligible and the main factor contributing to Δp at these pH

values is $\Delta\Psi$, with a value of 80mV (Kahn & Macnab, 1980). The presence of H+ ionophores (and DCCD) will collapse this $\Delta\Psi$, although at the concentrations of inhibitors used here it is likely that $\Delta\Psi$ will only be reduced slightly (Khan & Macnab, 1980; Hosoi et al., 1980).

Therefore it is possible to explain the increased efflux of chloroquine (1.7ug ml^{-1}) on the basis of electrical attraction; apart from the divalent chloroquine which is unable to pass back through the membrane, some monovalent chloroquine (+ve) may be held in vegetative cells by the $\Delta\Psi$ (inside -ve). When the $\Delta\Psi$ is lowered less of this monovalent chloroquine will be held and so is free to leave the cell. Presumably this effect is masked when large influxes of chloroquine occur because the ionophores would effectively increase the available H+ inside the cell and more chloroquine would be able to become doubly protonated and be bound inside. The DCCD results are difficult to explain but it is possible that DCCD increased the H+ levels inside the cell more than the ionophores and therefore more chloroquine would be able to become divalent so masking the increased efflux because of the lower $\Delta\Psi$.

Nevertheless metabolic inhibitors increase the accumulation of chloroquine in vegetative cells but not early outgrowing spores and it is probable that part of the reason that early outgrowing spores take up more chloroquine than vegetative cells is due a lack in early outgrowing spores of an effective energy-generating system which develops during outgrowth. Apart from the evidence provided by the metabolic inhibitors, the observation that at 4°C vegetative cells concentrate more chloroquine indicates that metabolic activity is required to lower chloroquine accumulation.

At pH 8 the mic for outgrowing spores and vegetative cells was the same (Chapter 6) yet although uptake of chloroquine was increased in both

cases at pH 8 vegetative cells did not concentrate chloroquine to the same extent as early outgrowing spores. The reasons for this anomaly are not clear, however it was observed that vegetative cells rapidly lowered the pH of the medium from pH8 to pH7.8-7.6; this would lower the amount of monovalent chloroquine present particularly near the pK (8.1). It was calculated that this would decrease the available monovalent chloroquine by at least 16%. This and a combination of volume errors and possible changes in volume with pH probably account for most of the difference and the significance of this result is doubtful.

If the outgrowing spore has a lower internal pH than vegetative cells, or less control over H+ flux than vegetative cells, the germinated spore would accumulate more chloroquine because the monoprotonated molecule would become doubly protonated and be effectively bound in the spore since the membrane is impermeable to the divalent ion. In vegetative cells less chloroquine would be in the divalent form and so could leave the cell. This could account for the preferential inhibition since the divalent chloroquine is more likely to bind to DNA (Cohen & Yielding, 1956b).

In support of this, early outgrowing spores exchanged less internal chloroquine with extracellular chloroquine than vegetative cells, and it was possible to mimic the decreased exchange of chloroquine in vegetative cells if the ionophore CCCP was present. There are several reports which show that spores have reduced cytochrome systems and lower ATPase activity than vegetative cells. These differences have been reviewed by Sussman and Halvorson (1966), basically spores contain little detectable cytochrome bands, and several associated enzyme activities are absent from spores e.g. NADH-cytochrome c-reductase and succinate-cytochrome c-reductase. However, spores contain four times as much soluble NADH oxidase as vegetative

cells and this is thought to oxidize NADH via flavinmononuclotide to oxygen unlike the usual vegetative sequence through membrane-bound cytochrome b, c1, c and a a_3 to oxygen. More recently Weber and Broadbent (1975) have shown that some enzymes associated with electron transport are present in differing amounts dependent on the method used to prepare the spores i.e. by medium exhaustion or resuspension. In B. megaterium vegetative cells contained four times more cytochromes a + a_3 and b than spores, also there was ten fold less ATPase activity in spore membranes (Ellar et al., 1975) Furthermore, ATPase activity increased during germination as did the activity of NADH oxidase in the absence of protein synthesis (Seto-Young & Ellar, 1979). The increase in activy may be due to activation of the enzymes during germination, since Tochikubo et al. (1978) have demonstrated that and inhibitor of soluble NADH oxidase is degraded during germination.

While there is species variation, in general it appears that spores have a reduced ability to carry out many vegetative electron transport functions and H+ translocations either by having reduced levels of enzyme or natural inhibitors present which are degraded during germination and outgrowth. This would explain why ionophores and other inhibitors did not increase chloroquine uptake in outgrowing spores, since there was no function to inhibit, and also why vegetative cells can increase the efflux of chloroquine by maintaining either a higher internal pH or H+ free cytoplasm unlike early outgrowing spores. There is further circumstantial evidence for this lack of electron transport activity in spores provided in a report by Walker et al. (1975). Outgrowing spores were resistant to a bacteriocin which uncoupled oxidative phosphorylation, but became sensitive about the time of first septation , therefore, unless spores lacked binding sites for the bacteriocin the mechanism of

oxidative phosphorylation is different in outgrowing spores (Walker et al., 1975). Also, dormant spores of B. cereus and B. megaterium have an internal pH of 6.3 and pH 6.38 respectively consistent with the hypothesis stated above, however on germination the pH rose to 7.3 (Setlow & Setlow, 1980). Nevertheless there was some variation in internal pH dependent on the external pH which may indicate a lack of ability to control internal pH as strictly as vegetative cells.

In summary, early outgrowing spores concentrate more chloroquine than vegetative cells or late outgrowing spores. Vegetative cells accumulate more chloroquine when treated with a variety of metabolic inhibitors. The level of chloroquine bound in both outgrowing spores and vegetative cells treated with CCCP, is consistent with an hypothesis that they accumulate more divalent chloroquine inside due to entry of the monoprotonated chloroquine and further dissociation to the membrane-insoluble doubly protonated form. It is likely that early outgrowing spores have either a low internal pH or an inability to promote efficient H+ flux and that they effectively bind more chloroquine than vegetative cells. Therefore, the basis of preferential uptake of chloroquine by outgrowing spores is their inability to cause efflux of the drug in the way that vegetative cells can.

The likely site of development of the function conferring resistance during outgrowth is the cell membrane. Thus one approach to finally pinpointing the biochemical function concerned in the differential sensitivity of spores to chloroquine (and presumably many other compounds) is to isolate resistant mutants and analyse their membrane proteins. This approach should ultimately provide useful mutants for analysing other aspects of development during outgrowth, including regulation. These aspects are examined in the next Chapter.

CHAPTER 8

Chloroquine-resistant mutants

Introduction

The differential uptake of chloroquine by spores may be caused by the absence of an energy-dependent efflux mechanism during early outgrowth. Some, at least, of the components of such a mechanism are likely to be present in the cell membrane since most of the electron transport functions and ATPase activity is membrane bound (Harold, 1972). Therefore, it is likely that some, if not all, mutants that are resistant to chloroquine have altered membranes since if chloroquine is binding to double stranded DNA a resistance mutation would probably be one affecting uptake (or exit) rather than an altered chromosome.

In this chapter the isolation of mutants resistant to chloroquine during vegetative growth is described and the characterisation of their membranes by SDS-gel electrophoresis is discussed.

Chloroquine resistant mutants

Extensive attempts to isolate mutants of *B. subtilis* resistant to chloroquine (500ug ml^{-1}) during outgrowth and sporulation were unsuccessful although several mutagens were used (UV, NTG and EMS). If as we have discussed previously, spores lack a mechanism for excluding the drug, this result is not surprising.

However, it was possible to isolate several spontaneous mutants resistant to 5mg ml^{-1} chloroquine during vegetative growth, one of which was chosen for further study (CQR1). The mutant was resistant in both rich and minimal media but growth in the presence of chloroquine (5mg ml^{-1}) was slow (Fig. 46) and the cells were of irregular shape.

All of the mutants including CQR1 grew normally under a variety of conditions and none were temperature sensitive for outgrowth, vegetative growth or sporulation. CQR1 also showed cross resistance for novobiocin during vegetative growth (Fig. 47).

The patterns of synergism seen with the metabolic inhibitors and chloroquine were identical when the mutant was compared with the wild type (data not shown); thus they were likely to be uptake mutants.

Outgrowth of CQR1 spores was still inhibited at the lower concentration of chloroquine (500ug ml^{-1}), this may indicate that the function altered in the mutant did not operate during outgrowth. However, after the cells had completed outgrowth and were divinding vegetatively they were not resistant to chloroquine (5mg ml^{-1}); the mutation was lost. All of the mutants including CQR1 were similar in this respect. Indeed, all the mutants rapidly reverted to the wild-type on pla with or without chloroquine present, and whole populations or single colonies lost resistance to chloroquine after subculturing several times. This observation may indicate that the gene(s) conferring resistance to chloroquine were carried on an unstable plasmid. However, there was no evidence that the resistance was conferred by a plasmid, since extracts of DNA from the mutant examined by agarose-gel electrophoresis showed no discrete low molecular mass bands (data not shown). Presumably the mutation was disadvantageous, however, by testing for resistance regularly it was possible to ensure that one was working with mutants rather than revertants to wild-type strains. Mutants isolated after mutagenesis with NTG were also unstable with respect to chloroquine resistance.

Since it was likely that the mutant was affected in its ability

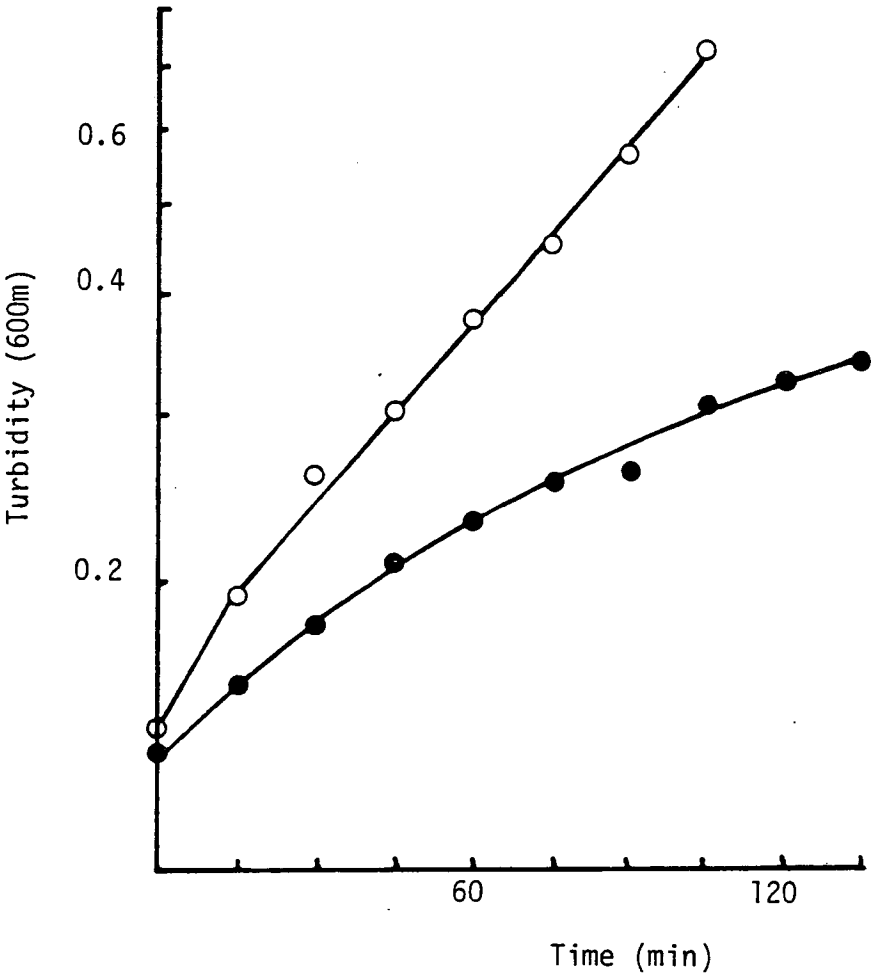


Figure 46. Growth of a chloroquine resistant mutant (CQR1). Vegetative cells of CQR1 were grown in NB: (○), no chloroquine; (●), 5mg ml^{-1} chloroquine added at zero time.

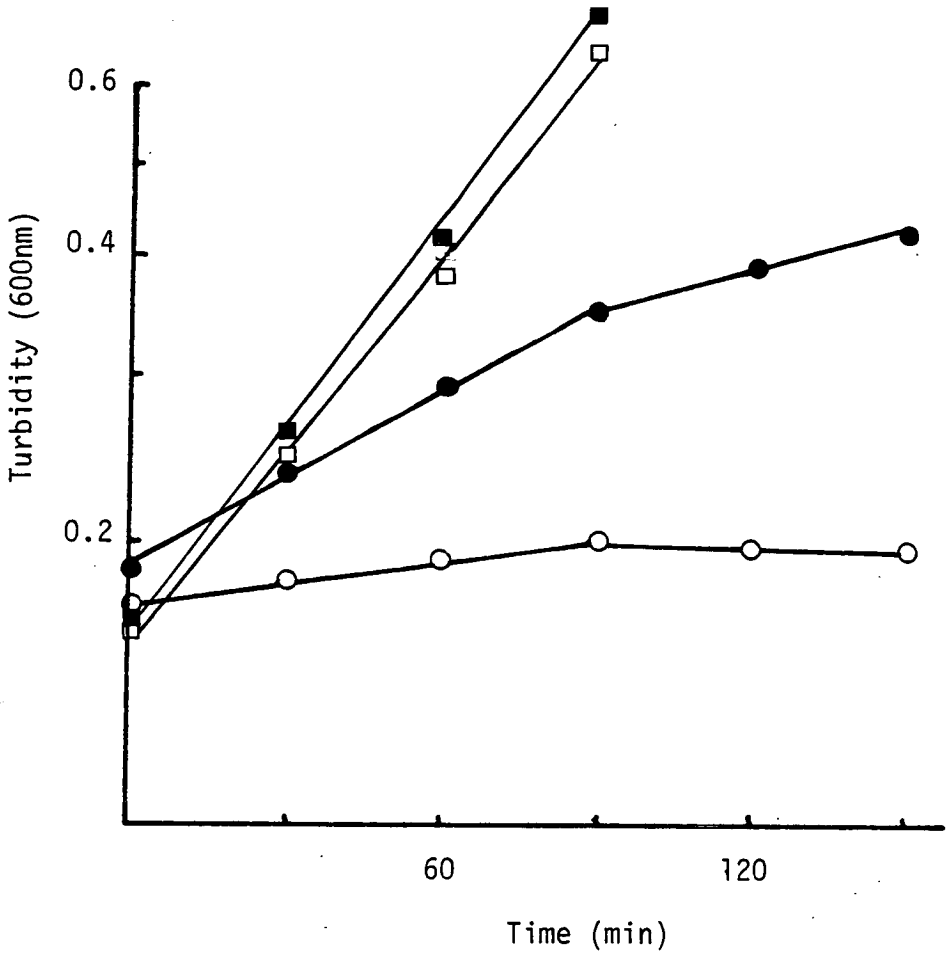


Figure 47. Effect of novobiocin on vegetative growth. Cells were grown in NB containing 6ug ml^{-1} novobiocin (○), wild type; (●), CQR1. No novobiocin; (□), wild type; (■), CQR1.

to concentrate chloroquine, the profile of membrane proteins of the mutant was compared with that of wild type vegetative cells.

Membrane changes in chloroquine resistant cells

The patterns of membrane proteins obtained by SDS-polyacrylamide gel-electrophoresis of isolated membranes from a chloroquine-resistant mutant (CQR1) and wild type vegetative cells were very similar (Plate 1). However, one protein with a molecular mass of 260,000 daltons (260K)* was present in increased amounts in CQR1 (indicated by the arrow). In minimal medium it was derepressed in both the wild type and CQR1 (Plate 2). Chloroquine did not induce synthesis of the protein (data not shown).

If this protein were directly involved in chloroquine resistance it might be expected that vegetative cells of the wild type grown in minimal medium would be more resistant than those grown in NB. This was not, however, found to be the case. Therefore, while this protein may play some role in the exclusion and entry of chloroquine, there are clearly other factors operating. For example, two of the inhibitors of energy metabolism (oxalate and rotenone) were only effective in rich medium.

This difference prompted an investigation of membrane changes during spore outgrowth. The profile of membrane proteins of dormant spores, seen by SDS-gel electrophoresis, was similar to that of vegetative cells although there was a significant difference in the levels of several proteins in the spore membrane. A line drawing of these differences is shown in Figure 48 (photographic reproduction of the gels was poor, especially of the less intense bands). Bands 4 and 6 were present in greater amounts in the membranes

* See Figure 47A facing Plate 1 for estimation of molecular mass.

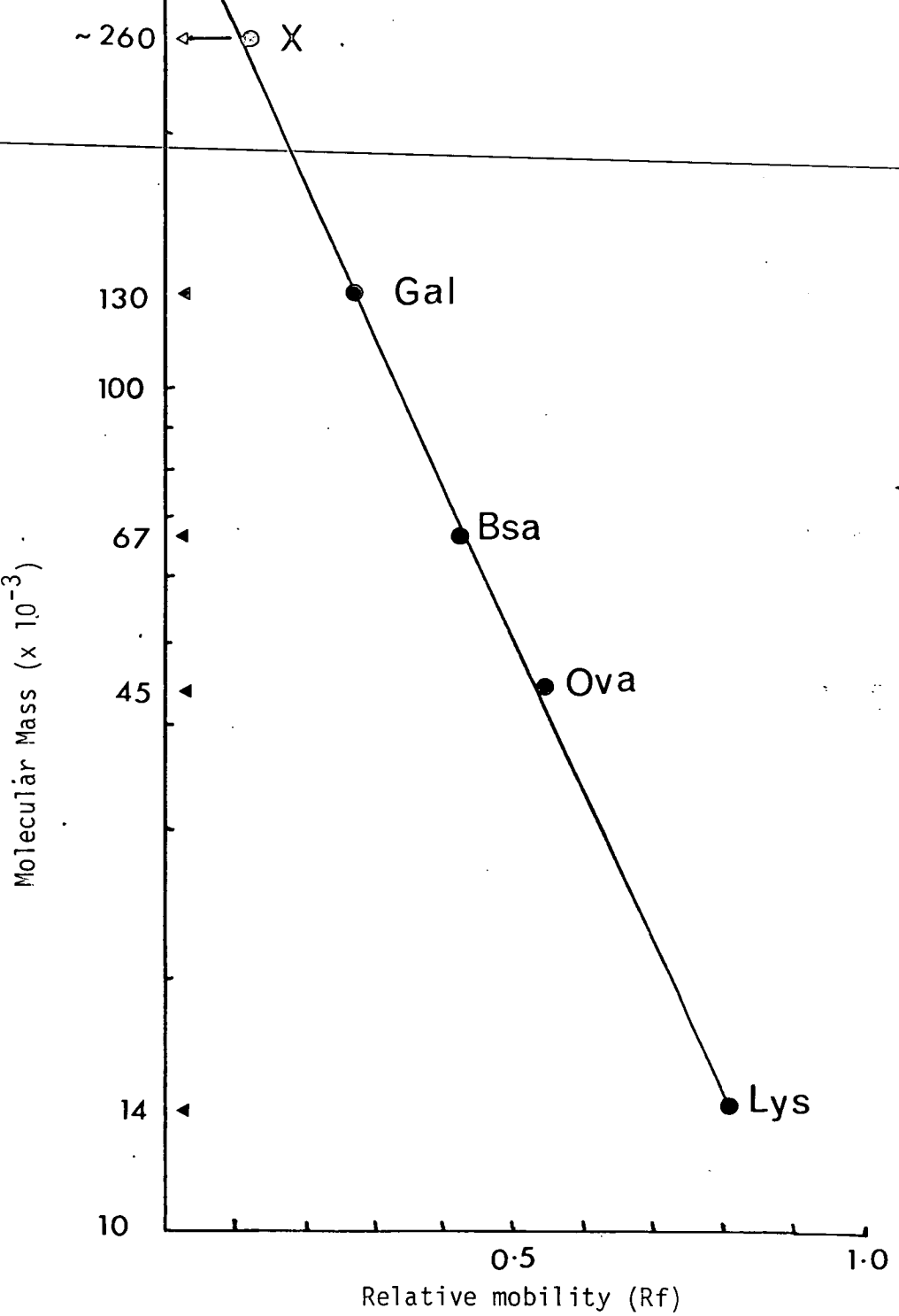


Figure 47A. Migration of SDS-dissociated membrane protein and standard proteins in SDS-polyacrylamide gels as a logarithmic function of molecular mass. Mutant membrane protein (X), standard protein (10ug of each), β -galactosidase (Gal), bovine serum albumin (Bsa), ovalbumin (Ova) and lysozyme (Lys) were electr phoresed under the conditions described in the methods. The migration of the polypeptides was measured on stained gels Rf values were calculated from the relative distance travelle by a protein compared to bromophenol blue and were the averag values from three separate gels. The molecular mass of X was estimated by extrapolation of the standard curve and mole mass read off the Y axis, a value of approximately 260000 daltons was obtained.

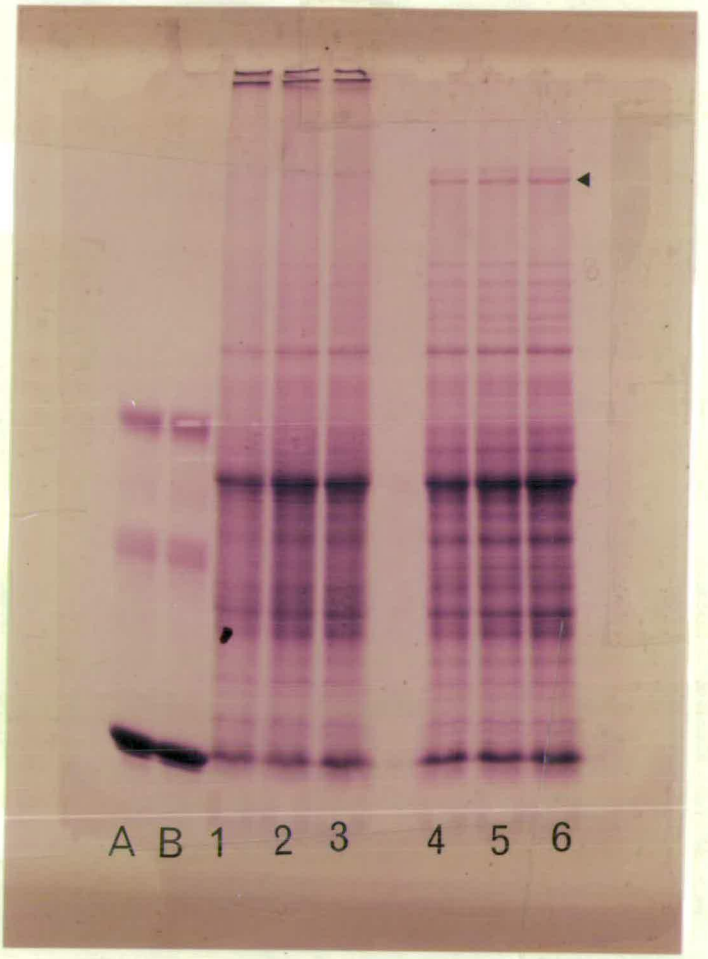


Plate 1. SDS-polyacrylamide gel-electrophoresis of membrane proteins. Cells were grown in NB and the membranes prepared by the method of Konings et al. (1973). Channels 1-3 are membranes from the wild type; channels 4-6 are membranes from the chloroquine resistant mutant. The arrow indicates the 260K protein. A and B are molecular mass markers, excluding Gal, see facing page for details.

Plate 2. SDS-polyacrylamide gel electrophoresis of membrane proteins. Vegetative cells were grown in NB or minimal medium, membranes were prepared as described previously in the methods. Channels 1 and 2 are cell membranes from the chloroquine resistant mutant grown in minimal medium and NB respectively; channels 3-4 are cell membranes from the wild type grown in minimal medium and NB respectively. The arrow indicates the 260K protein.

of dormant spores (Channel 2) and in vegetative cells grown in minimal medium, therefore their synthesis is probably derepressed during sporulation because of medium depletion. Bands 3 and 5 were absent in the membranes of dormant spores but appeared in the membrane during outgrowth (Channel 3 and 4). Bands 1 and 2 were faint in the membranes of dormant spores and developed during outgrowth. One of these (band 2) was the 260K polypeptide that was derepressed in the chloroquine-resistant mutants. Therefore it is possible that synthesis of this protein is required for the development of chloroquine resistance during spore outgrowth.

In the attempt to isolate stable mutations conferring chloroquine resistance, mutants resistant to quinacrine (a molecule of similar structure) were selected. These were cross resistant to chloroquine during vegetative growth, but they did not show the synergistic effect seen with combinations of chloroquine and the metabolic inhibitors and they had a membrane protein composition identical with that of the wild type. Therefore, it is possible that there are two separate functions that can confer resistance to chloroquine during vegetative growth. Spores prepared from the quinacrine-resistant mutants were sensitive to chloroquine (500ug ml^{-1}) during outgrowth (data not shown) but in this case the mutation was stable through sporulation and outgrowth. The resulting vegetative cells were resistant. Unfortunately it was not possible to test if the two mutations conferring resistance to chloroquine were linked since genetic analysis of CQR1, at least, would be very difficult because of the instability of the mutation.

Conclusions

The difficulty in obtaining mutants which are resistant to chloroquine during sporulation and spore outgrowth indicates that spores do not have any mechanism that can be altered to prevent accumulation of chloroquine or that such a mutation may confer asporogeny on any cell carrying it. Vegetative cells resistant to chloroquine (5mg ml^{-1}) sporulated normally and if the CQR1 mutation is related to the normal resistance of wild-type vegetative cells the latter possibility is unlikely, however, the instability of the CQR1 mutation complicates this observation.

The reason for the instability of the mutation conferring chloroquine resistance is unclear; it is possible that an unstable plasmid is involved in the resistance of vegetative cells but there was no evidence of such a plasmid in the mutant CQR1. The resistance function may be phenotypic rather than genotypic or the mutation may be subject to phenotypic suppression by environmental factors, however, a more detailed analysis of mutants resistant to chloroquine is required before any conclusions can be drawn.

The observation that the chloroquine resistant mutation conferred cross-resistance to novobiocin is particularly interesting because novobiocin and chloroquine maybe excluded by the same mechanism in vegetative cells and since both drugs are preferential inhibitors of outgrowth it is possible that the nature of this preferential inhibition is the same in both cases i.e. absence of a metabolic function during outgrowth. Two other Bacillus mutants resistant to streptomycin and ethidium bromide, due to reduced uptake of the drugs, were also only resistant during vegetative growth (Staal and Hoch, 1972; Bishop and

Brown, 1973). In the case of the mutant resistant to ethidium bromide the resistance during vegetative growth was temperature dependent and the authors concluded that the cell membrane was the probable site of the altered function (Bishop and Brown, 1973). The streptomycin-resistant mutant was only resistant in minimal medium containing glucose and had a defective cytochrome system (Staal and Hoch, 1972). Two conclusions can be drawn from these studies: first the function conferring resistance in both cases was not active during sporulation or spore outgrowth. Secondly, the function is probably related to energy metabolism or membrane function. Both of these would fit an hypothesis that early outgrowing spores lack certain energy related functions which would normally exclude or cause efflux of drugs. It would be interesting to test the susceptibility of thesemutants to chloroquine.

Quinacrine-resistant mutants (which were cross resistant to chloroquine) did not show the synergystic effect between chloroquine and the metabolic inhibitors observed in both wild-type cells and CQR1. Furthermore, the 260K membrane protein was not altered in the quinacrine-resistant mutant and the two mutations are probably unrelated. Therefore, there are two functions which can cause chloroquine resistance in vegetative cells, neither of which acts during outgrowth.

The membrane protein that was derepressed in vegetative cells resistant to chloroquine was present in very low amounts in dormant spores and was synthesised as outgrowth proceeded. It is possible that the extraction of the membranes of dormant spores was incomplete, but several different methods of extraction did not appear to release more of this protein. Furthermore, a pulse labelling study on proteins sythesized by

Figure 48. Diagrammatic representation of the main differences betwe vegetative cell membrane proteins and those of dormant and outgrowing spores. Membranes were prepared as described in the methods section. Track 1, vegetative cells; track 2, dormant spores; track 3 early outgrowing spores; and track 4, late outgrowing spores. Dotted lines represent very faint bands.

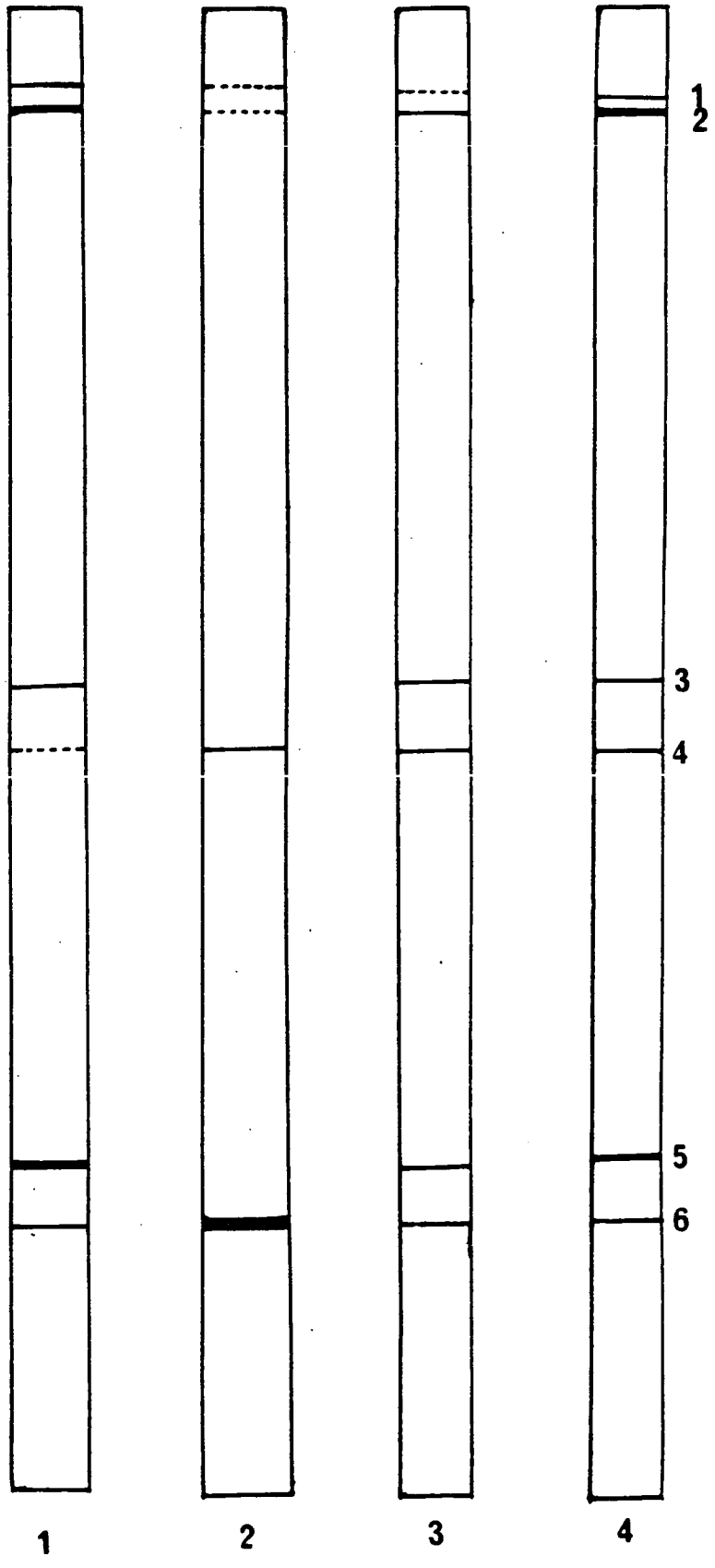


Figure 48. Diagrammatic representation of the main differences between vegetative cell membrane protein and those of dormant and outgrowing spores.

sporulating cells. Linn and Losick (1976) showed that two proteins of high molecular weight, similar to bands 1 and 2 shown in Figure 48 (track 1), including the 260K protein, were not synthesized in the later stages of sporulation. The low level of this protein in dormant spores and the subsequent increase as outgrowth continued is circumstantial evidence that it maybe involved in the development of chloroquine resistance during late outgrowth or at least is a marker event for the function conferring resistance to chloroquine during late outgrowth. Chloroquine resistance maybe conferred by other proteins in the membrane several of which differ in early outgrowing spores.

Seto-Young and Ellar (1979) have shown that several proteins are degraded during germination of B. megaterium spores and this may be associated with the increase in electron transport during germination (Wilkinson et al., 1977). B. megaterium spores are more suited for the study of spore membranes, since it is possible to protoplast the dormant spores. However, vegetative cell membranes of B. megaterium ATCC 9885 did not have the 260K protein, and the work was not continued because of insufficient time. Therefore, no conclusions can be drawn but presumably the preferential inhibition of outgrowth of B. megaterium spores by chloroquine is similar to that seen in B. subtilis

GENERAL DISCUSSION

General discussion

Detailed discussion of the results has been included at the end of each chapter and the results summarised in the abstract. In this section an hypothesis to account for the preferential inhibition of spore outgrowth by chloroquine will be presented and its relevance to spore outgrowth discussed.

The preferential inhibition of spore outgrowth by chloroquine is due to the inability of the early outgrowing spores to cause efflux of the drug by an energy-dependent mechanism. Therefore, early outgrowing spores accumulate more chloroquine than vegetative cells which are able to excrete the drug. The nature of this efflux mechanism may, therefore, be as shown in Figure 49. In the early outgrowing spore (Fig. 49, 1) there is a low internal pH (i.e. a source of protons), and lipid soluble monoprotonated chloroquine which is able to pass through the membrane, enters the protoplasm where is becomes doubly-protonated. The doubly-protonated chloroquine is insoluble in the lipid membrane and is effectively bound in the spore. In the case of vegetative cells (Fig. 49, 2) monoprotonated chloroquine enters the cytoplasm but because of electron transport and ATPase activity there are fewer free protons and so there is less doubly-protonated chloroquine formed, therefore, less chloroquine is bound. Vegetative cells treated with metabolic inhibitors are similar to early outgrowing spores (Fig. 49, 3), that is, there are protons available for the lipid soluble form of chloroquine to ionize to the lipid insoluble form and so be bound in the cell. The doubly-protonated form of the drug is thought to be the active fraction of chloroquine able to bind to DNA and proteins so inhibiting growth (see results Chapter4; Parker and Irvin, 1952; Cohen and Yielding, 1965b).

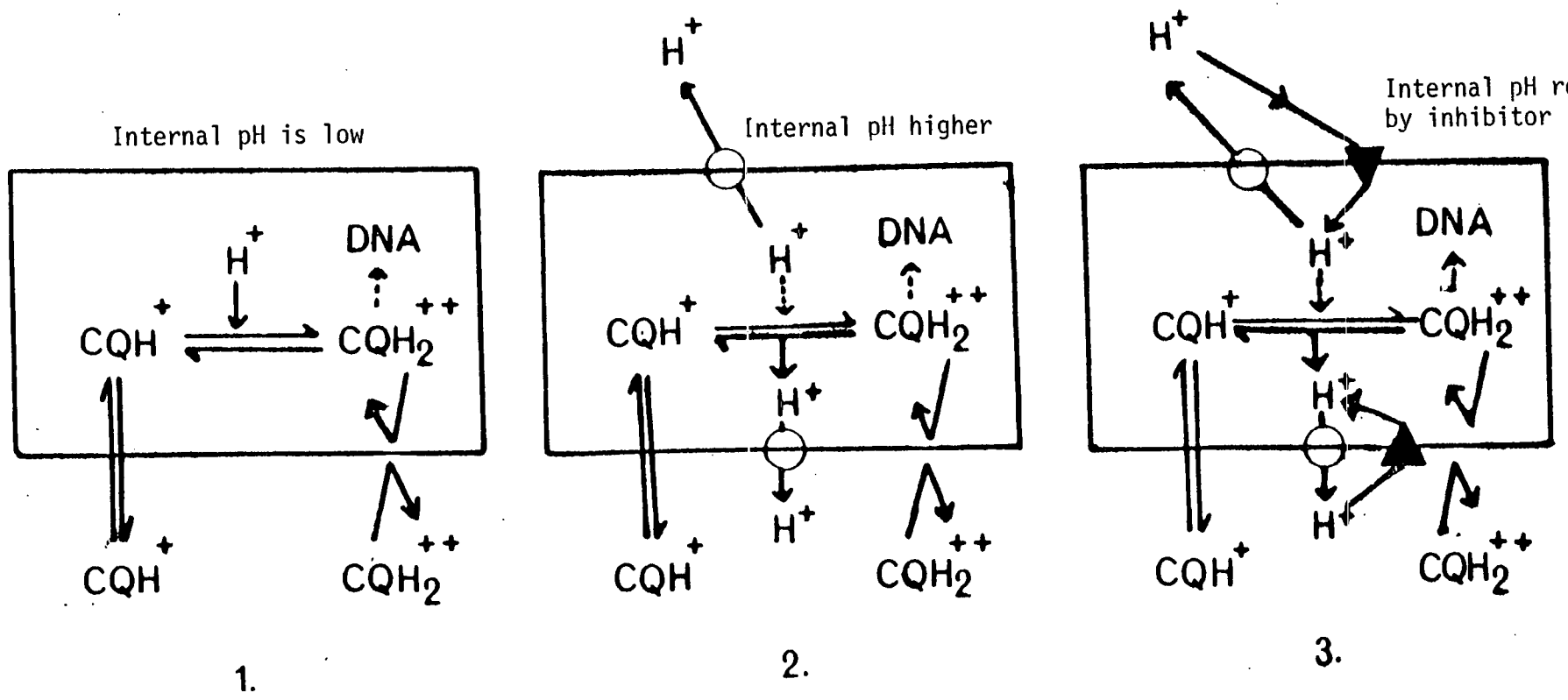


Figure 49. The mechanism of differential chloroquine accumulation.

1, early outgrowing spores; 2, vegetative cells; 3, vegetative cells treated with metabolic inhibitors. Abbreviations: CQ, chloroquine; H^+, proton; O electron transport (ATPase); ▲ metabolic inhibitor.

The outgrowing spore was sensitive to the lower concentration of chloroquine for only a brief period after the spore has germinated. Since resistance developed before large scale protein synthesis had started (Chapter 4 and 5) it is possible that either inhibitors of electron transport synthesized during sporulation were still present and were inactivated, or that the membrane conformation in spores differs from that of the vegetative cell (Tochikubo et al., 1978; Seto-Young and Ellar, 1979) and the conformation change activates the membrane during early outgrowth. It is also possible that precursors of electron transport proteins or ATPase particles formed during sporulation are incorporated in the membrane during outgrowth or that there is synthesis of a small amount of the protein concerned with chloroquine resistance ahead of the large scale protein synthesis. Development of resistance to chloroquine may be a marker event for one (or all) of these possibilities and a more detailed study of membrane activation during spore outgrowth may be fruitful in understanding membrane formation in general (see Chapter 8) and chloroquine resistant mutants should prove very useful in these studies.

Setlow and Setlow (1980) have suggested that a low internal pH in spores is involved in maintaining the dormancy of spores since enzyme activity would be reduced at low pH values. The phenomenon of low internal pH has also been reported in spores of Saccharomyces cerevisiae (Barton et al., 1980). If the pH remained low into outgrowth it may contribute to the low synthetic ability of early outgrowing spores. Furthermore outgrowing spores may not have a fully energized membrane and this would have several consequences for the outgrowing spore apart from an inability to maintain its internal pH. For instance,

uptake of many compounds would be affected as would be oxidative phosphorylation. It would be interesting to examine, in detail, some of the functions associated with an energized membrane during spore outgrowth; such an investigation may prove invaluable in understanding the interactions of various components of membrane function and structure and any possible specific adaptations to the lack of such membrane-linked functions made during outgrowth.

It appears that the significant changes in energy metabolism of the spore influence the accumulation of a preferential inhibitor of spore outgrowth (and sporulation?). Increased sensitivity to novobiocin during spore outgrowth has been used as evidence that there is a special requirement for DNA-gyrase activity during outgrowth (Gottfried et al., 1979), since mutation studies have indicated that DNA-gyrase is an important target for this drug. While this is an attractive hypothesis, it is much more likely that novobiocin is taken up more readily by outgrowing spores than by vegetative cells and this may be related to the energy-generating system in vegetative cells (Fig. 40). However, novobiocin binding in the cell would not be promoted by a low internal pH since the relationship between its lipid solubility and pH is the reverse of that seen in the case of chloroquine i.e. novobiocin is more lipid soluble at low pH. Nevertheless, there is no reason to suppose that novobiocin need be bound in the cell in the same way as chloroquine and it is possible that novobiocin is more effective in the lipid-soluble form. Clearly, caution is necessary when speculating on the significance of results obtained by drugs whose entry into the cell is influenced by factors unrelated to their mode of action.

Other molecules with a similar charge to chloroquine e.g. erythromycin are not preferential inhibitors of spore outgrowth. Therefore, preferential inhibition of spore outgrowth by drugs is a special case and there are a variety of complicating factors involved in the mechanisms by which antibiotics interact with cells.

The preferential inhibition of spore outgrowth by chloroquine is a marker event for a change during outgrowth of an important aspect of energy metabolism. Furthermore, it appears that a drug is actively excreted and this could be an important concept in drug resistance. A similar phenomenon has been reported by Kushmer and Khan (1968).

Future work

Several areas are open for further investigation on the basis of the results presented here. It would be interesting to examine in more detail the membrane (and any cytoplasmic) functions affecting chloroquine uptake that change during outgrowth and by selection for appropriate mutants to look at the regulation of these changes.

Furthermore, a general investigation of the internal pH and membrane potential during outgrowth would be valuable and spore outgrowth maybe a useful model system to examine the factors involved in the maintenance of an energized membrane from a structural and biochemical viewpoint.

Chloroquine can be used to isolate a range of useful mutants. For example in the course of this work mutants have neem isolated that undergo outgrowth faster than the wild type by selecting for more rapid escape from chloroquine inhibition, these may prove useful in

biochemical studies of spore outgrowth. A more general use of chloroquine would be in screening mutants affected in energy metabolism, on the basis of susceptibility and resistance to the drug.

APPENDIX

Platelet β-lysins

In this appendix the results of some work done in collaboration with Dr. J. Dawes[1] and Dr. D.S. Pepper[2] are presented (see abstract).

The aim of the collaboration was to identify the relationships between the bactericidal protein, β-lysin, which is active against Bacillus spp., and several other well characterised proteins released by platelets. I was also interested in defining the effects of β-lysin on spore germination and outgrowth as compared with its effects on vegetative growth.

There are three β-lysins in serum; these consist of lysozyme, β-lysin from platelets and β-lysin of non-platelet origin (Donaldson and Tew, 1977). I will consider only the platelet-derived enzyme. Its mode of action is unclear. The protein is thought to attach to the cell membrane, disrupt the respiratory chain, increase ATPase activity, and eventually cause lysis of the cell. This inhibitory mode is similar to the action of cationic detergents (β-lysin is a basic protein) but different β-lysin polypeptides are specific for different species of bacteria, unlike detergents (Donaldson and Tew, 1977).

The activity of β-lysin is usually assayed by measuring the number of bacteria killed after a brief incubation with the protein using a plate count method where one unit is equivalent to a 99% kill of total bacteria (Donaldson et al., 1964). However, this method is unwieldy where a large number of samples are to be assayed e.g. after column chromatography.

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during the purification of

Furthermore, bacteria are sensitive to many of the reagents used blood proteins e.g. Na azide, high NaCl concentrations etc. It was decided to assess the sensitivity of spore germination to β-lysin since germination can be measured quickly (30-60 min) and the germination process is relatively resistant to many of the reagents used in the purification compared to vegetative cells (data not shown).

Several fractions of human platelet concentrate eluted from a heparin agarose column with a NaCl gradient inhibited L-alanine germination of B. subtilis spores (Fig. 50). However, the two peaks of activity did not co-incide with the β-lysin activity measured by the plate count assay and the overall levels of inhibition were low. Other platelet purifications gave similar results and further assays were done using the plate count assay.

The bactericidal activity (β-lysin) of platelet-release proteins from rabbit platelets did not coincide with β-thromboglobulin (β-TG), platelet factor 4 (PF4) or platelet-derived growth factor and is therefore a separate protein species. β-lysin activity was also present, but at much lower levels, in human platelet release products, and again could be distinguished from β-TG, PF4 and growth factor activity.

The two peaks active against germinating spores in both human and rabbit release products correlated approximately with β-TG and PF4, and it would be interesting to characterise this inhibition further. In both cases germination was only inhibited to a maximum level of about 30-40% and increasing amounts of protein did not increase the level of inhibition.

A similar "plateau" of inhibition was observed using vantocil

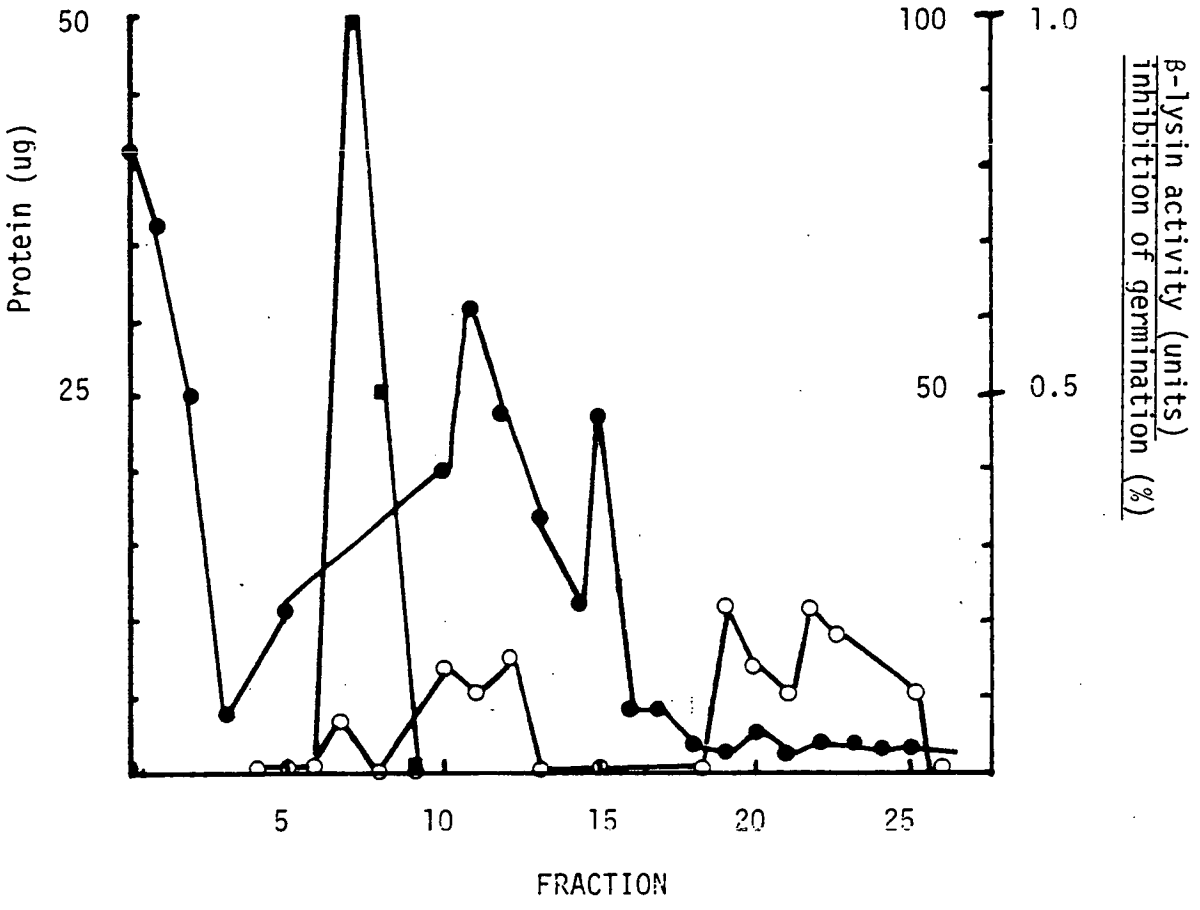

Figure 50 Fraction of platelet proteins. Human plateleted concentrate was eluted from a heparin-agarose column with a linear NaCl gradient (0-2M) in 15mM Na citrate buffer, pH 6.7, containing 3mM NaN3. 0.1ml samples were assayed for β-lysin activity by the method of (Donaldson et al.,(1964). Inhibition of germination in 0.1M Na-phosphate buffer pH 7.0 containing 10mM L-alanine, was estimated from the reduction in Δ turbidity (600nm) after 1h at 37°C. Fractions were diluted 1 in 4 with the germination mixture (●),protein (Bradfords dye binding assay); (○), inhibition of spore germination; (■), β-lysin activity

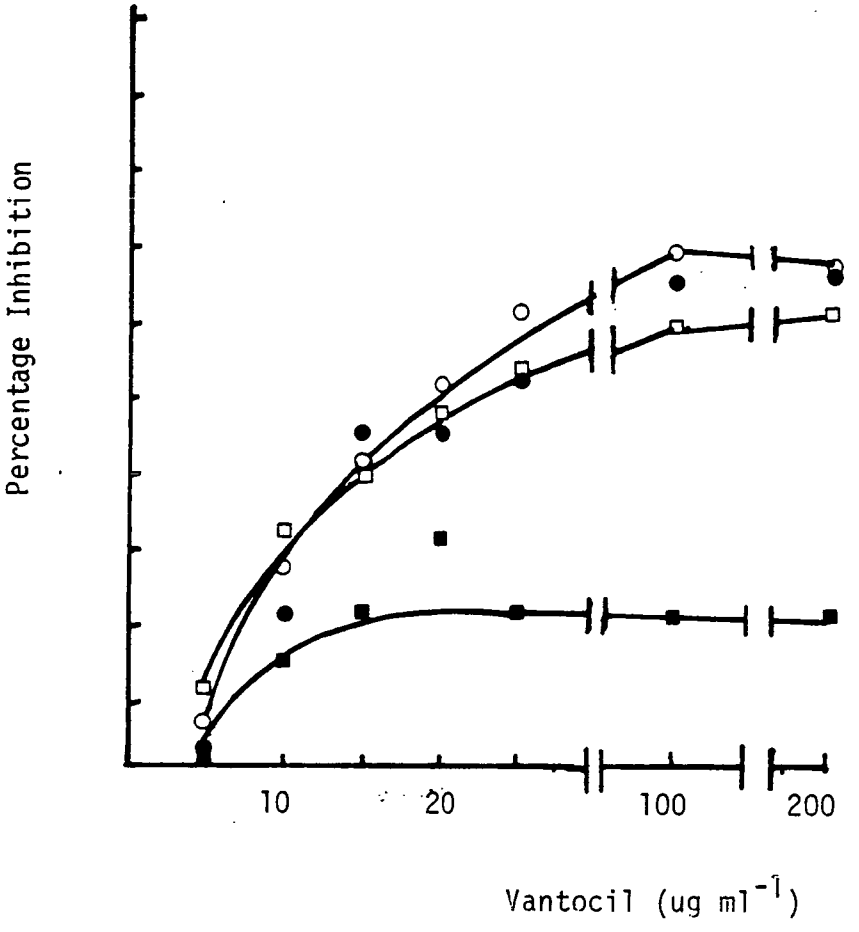

Figure 51. Effect of vantocil on spore germination. Inhibition of spore germination was measured as described in the legend of Fig. 51. (○), high molecular weight vantocil; (●) medium molecular weight vantocil; (□), low molecular weight vantocil; (■), branched vantocil.

(a polyarginine molecule, supposedly closely related to β-lysin) i.e. inhibition did not increase above a certain level (Fig. 51). Another interesting feature of the inhibition of germination by vantocil was the difference between the effectiveness of the various forms; inhibition was independent of molecular mass but a branched isomer was less effective in inhibiting germination (Fig. 51). This may indicate that there is some steric effect at the binding site, or the branched molecule may not be able to penetrate the spore coat. Such steric effects may have limited the action of platelet proteins in preventing germination. Vantocil may be a useful inhibitor in studies of germination since it was more effective against germination than vegetative cell growth or spore outgrowth (data not shown).

Neither β-lysin nor vantocil inhibited spore outgrowth (measured by plate counts) more or less effectively than vegetative growth. If the action of β-lysin on cells is primarily to disrupt the cytochrone system or influence ATPase activity one might expect spore outgrowth to be more resistant to the action of β-lysin since these functions are reduced during spore outgrowth (see Chapter 7). However, it is possible that βlysin does not prevent spore outgrowth per se but is bound to the membrane and acts on the vegetative cells subsequent to outgrowth. This would not be evident from the plate count assay which is dependent on the ability of cells to form colonies.

Further investigation of the effects of platelet proteins on both germination and spore outgrowth may be useful in investigating the state of the cell membrane during these processes.

I would like to thank Dr. J. Dawes for her constructive criticism

of this section, and both Dr. J. Dawes and Dr. D.S. Pepper for providing me with their unpublished results and for the column fractions containing β-lysin.

RABBIT PLATELET RELEASE PRODUCTS: A CAUTIONARY TALE

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(MRC Immunoassay Team, Edinburgh,[1] F. Hoffman-La Roche and Co. Ltd.,[2] BTS Headquarters Laboratory, Edinburgh[3] Department of Microbiology, Edinburgh[4]).

We have attempted to establish an animal model in which to examine in vivo platelet stimulation, particularly during the screening of new drugs. The rabbit is a convenient species for this type of study. Heparin affinity fractionation of rabbit platelet release products revealed a different elution pattern from that obtained with human platelets. At least 4 separate proteins were identified; one of these may be termed rabbit PF4 but none was clearly equivalent to human β-TG. One protein, PRP1, eluted at 0.5M NaCl; a second, PRP2, at 0.8-0.9M NaCl. A radiommunoassay was developed to measure PRP2, which is present in rabbit plasma at 8-26 ng. ml^{-1}, and in serum at 2.9-3.5 ug. ml^{-1}. Its immunological cross-reactivity with human β-TG was minimal and it did not cross-react with human PF4. Its physical properties and amino acid sequence are being examined. A third peak was identified as the inhibitor of bacterial growth, β-lysin. β-lysin activity was also present, but at much lower levels, in human platelet release products, where it could be distinguished from β-TG, PF4 and growth factor activity.

It appears that evolution of the platelet release proteins has been recent. Each mammalian species possesses a family of such proteins which are closely related to one another, but cannot be identified with individual proteins from another species. Purification procedures developed for one mammal cannot, therefore, be applied indiscriminately to another, but once the proteins have been identified they are valuable indicators of platelet release in that species.

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ABSTRACTS

British Spore Group Meeting, Cambridge September, 1979

2.35 Preferential Inhibition of Outgrowth in *Bacillus subtilis* Spores by Chloroquine.

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The antibiotic, chloroquine, has been shown to preferentially inhibit spore outgrowth of *Bacillus subtilis* 168 (McDonald, W.C. (1967) Canadian Journal of Microbiology, 13, 611-613). Outgrowing spores escape from chloroquine inhibition after initiation of macromolecular biosynthesis. Inhibition of outgrowing spores and vegetative cells is relieved by divalent cations. Enzyme activity modulated by Mg^{2+} shows no change in the presence of chloroquine. Ethidium bromide exhibits a synergystic action with chloroquine. Studies with a mutant of *B. subtilis* 168, temperature-sensitive for initiation of DNA synthesis, show that levels of chloroquine not normally inhibitory to vegetative cells, completely inhibits further growth of the mutant on completion of chromosome replication. Sporulation is inhibited at all stages by low levels of chloroquine. On the basis of these results and experiments with DNA-dependent RNA polymerase in permeabilized cells, a hypothesis to account for preferential inhibition by chloroquine will be discussed.

Eighth International Spore Conference
October 9-12, 1980

Marine Biological Laboratory
Woods Hole, Massachusetts 02543

Preferential Inhibition of Bacillus subtilis 168 Spore Outgrowth by Chloroquine and Novobiocin. Evidence for Membrane Changes during Outgrowth.

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Chloroquine and novobiocin prevent B. subtilis spore outgrowth at lower concentrations than those inhibiting vegetative growth. At pH levels where both antibiotics are more lipophilic, pH 8 and pH 6 respectively, there is no preferential inhibition. In the presence of inhibitors of electron transport the preferential inhibition is not apparent, the vegetative cells are as sensitive as outgrowing spores. A mutant resistant to chloroquine during vegetative growth was also novobiocin resistant although the antibiotics within the cell act on different targets. A high molecular weight membrane protein has been shown by SDS gel-electrophoresis to be derepressed in this mutant. The escape time of outgrowing spores from chloroquine inhibition has been correlated to the appearance of membrane proteins during spore outgrowth. It is proposed that the preferential inhibition of outgrowth is the result of an effect on drug uptake and that there are during outgrowth specific changes in the cell membrane. Further to this, during vegetative growth a mechanism for excluding these antibiotics exists that is dependent on a fully functional electron transport activity and this is less active during early outgrowth.